The development of tolerance to nicotine's effects on anxiety in the rat.

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THE DEVELOPMENT OF TOLERANCE TO NICOTINE'S EFFECTS ON ANXIETY IN THE RAT

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June 2001

Thesis submitted for the degree of Doctor of Philosophy
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ABSTRACT

This thesis demonstrates many of factors that influence the effects of nicotine on anxiety. In the social interaction test, nicotine induced both anxiolytic and anxiogenic effects in singly housed animals that were dose-dependent, low doses having anxiolytic and high doses anxiogenic effects. However, in group-housed rats only anxiolytic effects were detected but at a more limited dose range. In singly housed animals, the effect of a low dose of nicotine (0.1 mg/kg; s.c.) was time-dependent with an anxiogenic effect observed after 5 min, an anxiolytic effect after 30 min and another anxiogenic effect after 60 min. After 7 days of nicotine treatment with this low dose, tolerance developed to the initial anxiogenic and the anxiolytic effect. The dorsal hippocampus and dorsal raphé nucleus (DRN) were identified as brain regions mediating these effects. After 7 days of nicotine treatment an anxiogenic withdrawal response was observed at 72h after termination of treatment and was reversed by nicotine injected subcutaneously or into the DRN. Animals trained to self-administer nicotine (0.45 mg/kg/day) for 4 weeks showed an anxiogenic effect as did rats receiving the same dose by intravenous injection, subcutaneous injection or continuous infusion by osmotic minipump. None of these animals showed a withdrawal response at 24 or 72h following termination of treatment.

In the elevated plus-maze, acute administration of nicotine had only anxiogenic effects, but the time-course of these effects were influenced by the housing conditions, with an

anxiogenic effect at 5 and 30 min in group housed rats, but 30 and 60 min in isolated rats. In isolated rats, 5 min after injection nicotine was ineffective but after 7 days of treatment (0.1 mg/kg/day) a significant anxiolytic effect emerged to which the rats became tolerant to after 14 days. An anxiogenic withdrawal response was observed at 24h after termination of nicotine treatment and was reversed by intra-hippocampal nicotine.

PUBLICATIONS ARISING FROM THIS THESIS

Irvine EE, Cheeta S and File SE (1999) Time-course changes in the social interaction test of anxiety following acute and chronic administration of nicotine. *Behav Pharmacol* 10:691-697.

Irvine EE, Cheeta S and File SE (2001) Tolerance to nicotine's effects in the elevated plus-maze and increased anxiety during withdrawal. *Pharmacol Biochem Behav* 68:319-325.

Cheeta S, Irvine EE, Kenny PJ and File SE (2001) Social isolation modifies nicotine's effects in animal tests of anxiety. *Br J Pharmacol* 132:1389-1395.

Irvine EE, Bagnalasta M, Marcon C, Motta C, Tessari M, File SE and Chiamulera C (2001) Nicotine self-administration and withdrawal: modulation of anxiety in the social interaction test in rats. *Psychopharmacology* 153:315-320.

Irvine EE, Cheeta S, Marshall M and File SE (2001) Different treatment regimens and the development of tolerance to nicotine's anxiogenic effects. *Pharmacol Biochem Behav* 68:769-776.

Cheeta S, Irvine EE, Kenny PJ and File SE (2001) The dorsal raphé nucleus is a crucial structure mediating nicotine's anxiolytic effects and the development of tolerance and withdrawal responses. *Psychopharmacology* 155:78-85.

Irvine EE, Cheeta S and File SE (2001) Development of tolerance to nicotine's anxiogenic effect. *Brain Res* 894:95-100.

Irvine EE, Cheeta S Lovelock C and File SE (2001) Tolerance to midazolam's anxiolytic effects after short-term nicotine treatment. *Neuropharmacology* 40:710-716.

ACKNOWLEDGEMENTS

Firstly, I would like to thank my supervisor, Prof Sandra File, for giving me the opportunity to work on such an exciting project for the last three years. Her enthusiasm and guidance have been invaluable. Thanks must also go to Dr Paul Francis and Dr Stephen Minger for all their help and advice.

A very special thank you must go to Dr Survjit Cheeta for the unique support and encouragement she has provided throughout my PhD. Also, I would like to thank her for making my time in the lab unforgettable and for being such a special friend. Thanks to Joy Heard and Dr Sonia Tucci for all their help and for keeping me entertained over the last few months. I would also like to thank all the people who I have also worked with in the lab, Dr Paul Kenny, Dr Emma Fluck, Dr Pallab Seth, Dr Nick Jarrett, Dr Cathy Fernandes and Dr Abdel Ouagazzal.

As part of my PhD I spent 3 months working at GlaxoWellcome in Verona and I would like to thank many of the staff that worked there during my stay. My deepest thanks to Dr. Christian Chiamulera for his support and direction, and for making my time in Italy so enjoyable. I would also like to thank Michela Tessari, Christina Motta, Christina Macron and Michela Bagnalasta for introducing me to self-administration and for making it such a friendly working environment. Also, thanks to Dr. Phil Gerrard who provided help in the discussion and conduction of some of the experiments

Finally a big thank you must go to my family and all my friends for believing in me and for the encouragement they have provided.

Elaine E Irvine

8/6/2001

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LIST OF ABBREVIATIONS

ACh	Acetylcholine
aCSF	Artificial cerebrospinal fluid
ANOVA	Analysis of variance
ANCOVA	Analysis of covariance
BDZ	Benzodiazepine
α-Bgt	α-bungarotoxin
cpm	Counts per minute
DHβE	Dihydro-β-erythroidine
DMPP	1,1-Dimethyl-4-phenyl-piperazinium
DRN	Dorsal raphé nucleus
EDTA	Ethylenediaminetetraacetic acid
GABA	γ-aminobutyric acid
GAD	Generalised anxiety disorder
5-HT	5-Hydroxytryptamine
[^{3}H]-5-HT	5-Hydroxy[G^{3}H]tryptamine creatine sulphate
HF	High light, familiar
i.p.	Intraperitoneal
i.v.	Intravenous
LU	Low light, unfamiliar
MLA	Methylylcaconitine
MRN	Median raphé nucleus
NA	Noradrenaline
nAChR	Nicotinic acetylcholine receptor
(-)-nicotine	[-]-1-Methyl-2-[3-pyridyl]pyrrolidine
8-OH-DPAT	(±)-8-Hydroxy-dipropilamino tetralin
PAG	Periaquaeductal grey
Pargyline	N-Methyl-N-2-propnylbenzylamine hydrochloride
mRNA	Messenger ribonucleic acid

sem	Standard error of the mean
SSRI	Selective-serotonin reuptake inhibitor
WAY 100,635	N-{2-[4-(2-Methoxyphenyl)-1-piperazinyl]ethyl}-N-2-pyridinyl)cyclohexanecarboxamine trichloride

CHAPTER 1

General Introduction

1.1 Anxiety

Anxiety can be defined as an emotional state that produces an unpleasant feeling of apprehension, which is normally accompanied by somatic symptoms and signs, including palpitations, hyperventilation, pallor, dry mouth, sweating, postural tremor, fatigue and tics (Millet et al., 1998). Anxiety and fear are very similar phenomena, but they can be distinguished from each other in that fear is related to a real threat, whereas anxiety is a response that occurs to the anticipation of danger (Bremner et al., 1996). Anxiety is a very common human experience and many people suffer from attacks daily, due to problems at work or social interaction. However, for 5-10% of the population, the anxiety is extreme and they feel the need to seek psychiatric help. The psychiatric diagnosis of an anxiety disorder is not a simple one, but there is broad agreement about major categories (American Psychiatric Association, DSM-IV, 1994; World Health Organization, ICD-10, 1993). In adults, anxiety disorders are divided into two subsections: anxiety states and phobic disorders (see Table 1.1). The treatment of these disorders is very complex as they are not equally drug-responsive and indeed different treatments are effective in treating the different types of anxiety. For example, Generalised anxiety disorder (GAD), the most common of the anxiety disorders, is treated most effectively with the benzodiazepines and 5-HT_{1A} receptor

agonists, whereas panic disorder responds better to antidepressant treatment. These differences in the treatment for anxiety disorders raise the question as to whether there are distinct neurobiological substrates that underlie the different types of anxiety.

Table 1.1 Classification of common anxiety disorders and their characteristics.

ANXIETY DISORDERS	
Disorder	**Characteristics**
Anxiety States	
Generalised Anxiety Disorder	Characterised by 6 months of anxious mood or worried preoccupations
Panic Disorder	Characterised by random unpredictable attacks of panic caused by a fear of dying for example
Obsessive-compulsive Disorder	Characterised by recurrent obsessions (thoughts, ideas, images, impulses) and compulsions (hand washing which serves to reduce anxiety)
Post-traumatic Stress Disorder	Characterised by re-experiencing a traumatic event accompanied with anxiety- and depressive-like experiences
Phobic States	
Social Phobia	Characterised by the persistent fear of situations in which the individual is exposed to the scrutiny of others
Simple Phobia	Characterised by intense anxiety when exposed to some specific phobic stimulus, and persistent fear of this stimulus between episodes of exposure
Agoraphobia	Characterised by an attack of anxiety or panic in a variety of public places

1.2 Drug Treatment of Anxiety

The benzodiazepines (e.g. diazepam [Valium] and chlordiazepoxide [Librium]) are a class of compounds that have commonly used in the treatment of anxiety disorders (File, 1990a; Lader, 1989). Benzodiazepines have a rapid onset of action and have a maximal effect within 2 weeks (Ashton, 1994). They have been most extensively used for the treatment of GAD (Argyropoulos et al., 2000; Ninan, 1999). Andresch et al. (1991) have shown alprazolam, a high potency benzodiazepine, to be effective in the treatment of panic disorder. However, another study showed that there was no significant difference on symptoms between the use of alprazolam and behavioural therapy (Klosko et al., 1990). The treatment of choice for specific phobic disorders is behavioural therapy but it has been suggested that the benzodiazepines may have an additive effect (Wardle, 1990). Benzodiazepines replaced barbiturates in the 1960s as they were thought to have fewer side effects, but over the last few years the unwanted side effects on the benzodiazepines have become apparent. Benzodiazepines have a number of adverse side-effects including psychomotor depression (Weingartner et al., 1993), cognitive impairment (Sellal et al., 1992; Lister, 1985) and long-term use may result in dependence (Rickels et al., 1988). There is now considerable evidence suggesting that with daily use, tolerance develops to the pharmacological effects of benzodiazepines, most noticeably to their sedative and anticonvulsant effects but also to their anxiolytic effects (Higgitt et al., 1988; Lucki et al., 1986). Withdrawal from these drugs also results in a syndrome that is often characterised in humans by symptoms such as insomnia, weight loss, increased anxiety, tremors and seizures (Tyrer, 1988; Ladewig, 1984; Pertusson and Lader, 1981). These unwanted side

effects have led to the search for novel compounds that are effective in treating anxiety, but do not have these side effects.

The serotonergic system has been implicated in the neurochemistry of anxiety for a long time and compounds selective for the 5-HT_{1A} receptors, such as buspirone, gepirone and ipsapirone, have been shown to have clinical efficacy in the treatment of GAD, without the apparent unwanted side effects of the benzodiazepines (Pecknold, 1997). However, in contrast to the immediate effects of the benzodiazepines, the onset of action of buspirone does not occur for two weeks and in some patients can take 3-6 weeks to take effect (Sussman, 1987). Also, patients who have responded well to benzodiazepines in the past appear to not respond so well to buspirone (Sussman, 1987). There is also evidence that 5-HT_2 receptor antagonists, such as ritanserin, have anxiolytic effects in humans (Katz, 1993; Bersani et al., 1991; Hensman et al., 1991; Bressa et al., 1987).

More recent studies have shown that many anxiety disorders can be treated with antidepressants. The selective serotonin re-uptake inhibitors (e.g. velafaxine and fluoxetine) have been shown to be effective in the treatment of most anxiety disorders (Argyropoulos et al., 2000; Schatzberg, 2000). The tricyclic antidepressants (TCAs), such as imipramine and clomipramine, have been shown to be therapeutic most noticeably in the treatment of panic disorder (Barlow et al., 2000). However, they are also effective in the treatment of GAD, obsessive compulsive disorder and like the benzodiazepines they have an additive effect to behavioural therapy in the treatment of

specific phobias (Argyropoulos et al., 2000). Monoamineoxidase inhibitors, such as phenelzine, have been shown to be as effective as the TCAs in the treatment of panic disorder but there use is restricted due to the side-effects in long-term use (Argyropoulos et al., 2000).

1.3 Animal Tests of Anxiety

Animal models of anxiety form the backbone of preclinical research and they endeavour to represent some aspect of the aetiology, symptomatology or treatment of anxiety disorders (Menard and Treit, 1999). There is a very wide range of animal tests of anxiety, which can be divided in to two main types, conditioned, and unconditioned behaviours, see Table 1.2. Models of conditioned behaviour involves animals' conditioned responses to stressful and often painful stimuli (e.g. exposure to electric footshock), whilst in unconditioned tests involve animals' spontaneous or natural reactions to stress stimuli that do not explicitly involve pain or discomfort (e.g. exposure to a novel test chamber).

Table 1.2 Some commonly used animal models of anxiety. Modified from Olivier et al., 2000.

Conditioned Responses	Unconditioned Responses
Conflict tests – Geller-Seifter conflict	Social interaction
Vogel punished drinking	
Fear potentiated startle	Elevated plus-maze
Shock-probe avoidance	Light-dark exploration
Conditioned taste aversion	Ultrasonic vocalisation

The models of conditioned behaviour that are most commonly used are the Geller-Seifter conflict test (Geller and Seifter, 1960) and the Vogel water-lick conflict test (Vogel et al, 1971). In the Geller-Seifter conflict test, lever pressing following a distinct stimulus (e.g. light) results in the presentation of food and a punishment (e.g. electric shock). Thus, the animal is faced with the choice between reward and punishment, which will result in a suppressed response. A variety of anxiolytic drugs produce an attenuation of the behavioural suppression, which is considered an indicator for anxiolytic activity in this model (Corbin et al., 2000; Giusti et al., 1993). The Vogel conflict model is a variation of this procedure but is more advantageous as it eliminates the prolonged training that the Geller-Seifter test needs (Vogel et al., 1971). In this test, rats are given shocks while drinking and administration of drugs that have been shown to be anxiolytic in humans attenuates this suppressed behaviour. Disadvantages of these tests are that it is difficult to know whether the anxiolytic effect is a true effect of the drug (e.g benzodiazepines) or to some other aspect of the drug's pharmacological effect, such as facilitation of their motivation for food, sedation and/or

reduced pain sensitivity. In addition, it is difficult to observe the anxiogenic effect of a drug because the punishment induced anxiety is at such a high level.

The second types of anxiety test are the unconditioned or ethologically based tests and these can be divided into tests that are based on social or exploratory behaviour. In contrast to the conditioned tests, the fear seen in these tests is not conditioned but produced by rats being in a novel environment. Three of the most widely used of the unconditioned tests are, the social interaction test (a test of social behaviour; Figure 1.1), the elevated plus-maze test (a test of exploratory behaviour; Figure 1.2) and the light-dark exploration test. The social interaction test of anxiety has been validated extensively using both behavioural and physiological measures (File and Hyde, 1978). In this test, the dependent variable is the time spent in social interaction by pairs of male rats, see Figure 1.1. A decrease in social interaction, without a concomitant decrease in locomotor activity, is defined as a specific anxiogenic effect and an increase in social interaction, without an increase in locomotor activity, is defined as an anxiolytic effect. The anxiety generated by this test can be manipulated by changing the light level in the test arena and/or the rats' familiarity with the arena. Social interaction is maximal when the animals are tested in an arena with which they are familiar and in which the light level is low. If the illuminance is increased, or if the arena is unfamiliar, the time spent in social interaction decreases, and is lowest when the rats are tested in an unfamiliar brightly lit arena. For a more detailed account of this test, see File (1997a). The social interaction test has proved sensitive to a wide range of anxiolytic drugs, such as the benzodiazepines, the barbiturates, ethanol,

selective serotonin re-uptake inhibitor, paroxetine, and the neurokinin$_1$ receptor antagonist, CGP 49823 (File, 1997b; Lightowler et al., 1994; Barnes et al., 1990; File, 1980; File et al., 1976). It is also possible using this test to detect anxiogenic effects of drugs, such as β-carbolines, pentylenetetrazole, picrotoxin, phencyclidine, caffeine, yohimbine and amphetamine (Buczek et al., 1998; Bhattacharya et al., 1997; Sams-Dodd, 1995; Johnston and File, 1988; File et al., 1986; Pellow and File, 1986; Guy and Gardner, 1985; File and Lister, 1984) and of neuropeptides such as ACTH and CRF (Sajdyk et al., 1999; Dunn and File, 1987; Niesink and van Ree, 1984; File and Clarke, 1980; File and Vellucci, 1978). The anxiogenic state that is induced by withdrawal from chronic treatment of benzodiazepines, ethanol, and caffeine can also be detected using this test (Andrews et al., 1997; Bhattacharya et al., 1997; File et al., 1992, 1991a, b; Andrews and File, 1992; Costall et al., 1990a, b; File et al., 1989). The test is also sensitive to an environmental stressors, such as the disturbance from building work (File, 1994) and to non-pharmacological methods such as a 5 min exposure to the odour of a cat (Zangrossi and File, 1992).

The elevated plus-maze apparatus is raised 50cm off the floor and consists of two open arms and two arms enclosed by walls. Rats normally avoid the two open arms and restrict their activity to the closed arms. An anxiolytic effect is indicated by an increase in the proportion of activity that is seen on the open arms (i.e. an increase in the percentage time spent on the open arms, and the number of entries on to the open arms), and an anxiogenic effect by a decrease in the portion of activity on the open arms. The number of times an animal enters in to the closed arms is the measure of

non-specific locomotor activity. For a more detailed account, see File (1992) and Pellow et al. (1985). Like the social interaction test, this test has proved sensitive to both anxiolytic (Hale et al., 1990; Johnston and File, 1989a) and anxiogenic (Cruz et al., 1994; Lapin, 1993; Johnston and File, 1989b; Baldwin et al., 1989) drugs. The anxiogenic state that is induced by withdrawal from chronic treatment of benzodiazepines and ethanol can also be detected using this test (File et al., 1991b, 1987a).

In the light-dark exploration test the number of transitions made by mice between a light and a dark compartment is used as the measure of anxiety (Crawley et al., 1994; Crawley, 1981). The mice are faced in this test with a conflict between the desire to explore a novel area and their aversion to bright light. An increase in transitions, without an increase in locomotor activity, is taken to indicate an anxiolytic effect. Mice are placed on the brightly lit side of a two-compartment chamber, and the number of transitions between the light and dark sides, as well as the time spent on the two sides are recorded. This test has also proved sensitive to both anxiolytic (Hascoët et al., 2000; Okuyama et al., 1999; Artaiz et al, 1998; Chaouloff et al, 1997; Bourin et al., 1996; Shimada et al., 1995; Barnes et al., 1992; Onaivi and Martin, 1989; Costall et al., 1989a, b, 1988) and anxiogenic (El Yacoubi et al., 2000; Shimada et al., 1995; de Angelis, 1992; Onaivi and Martin, 1989; Costall et al., 1989a, b) drugs. The anxiogenic state that is induced by withdrawal from chronic treatment of benzodiazepines, ethanol cocaine and nicotine can also be detected using this test (Costall et al., 1990a, b, c)

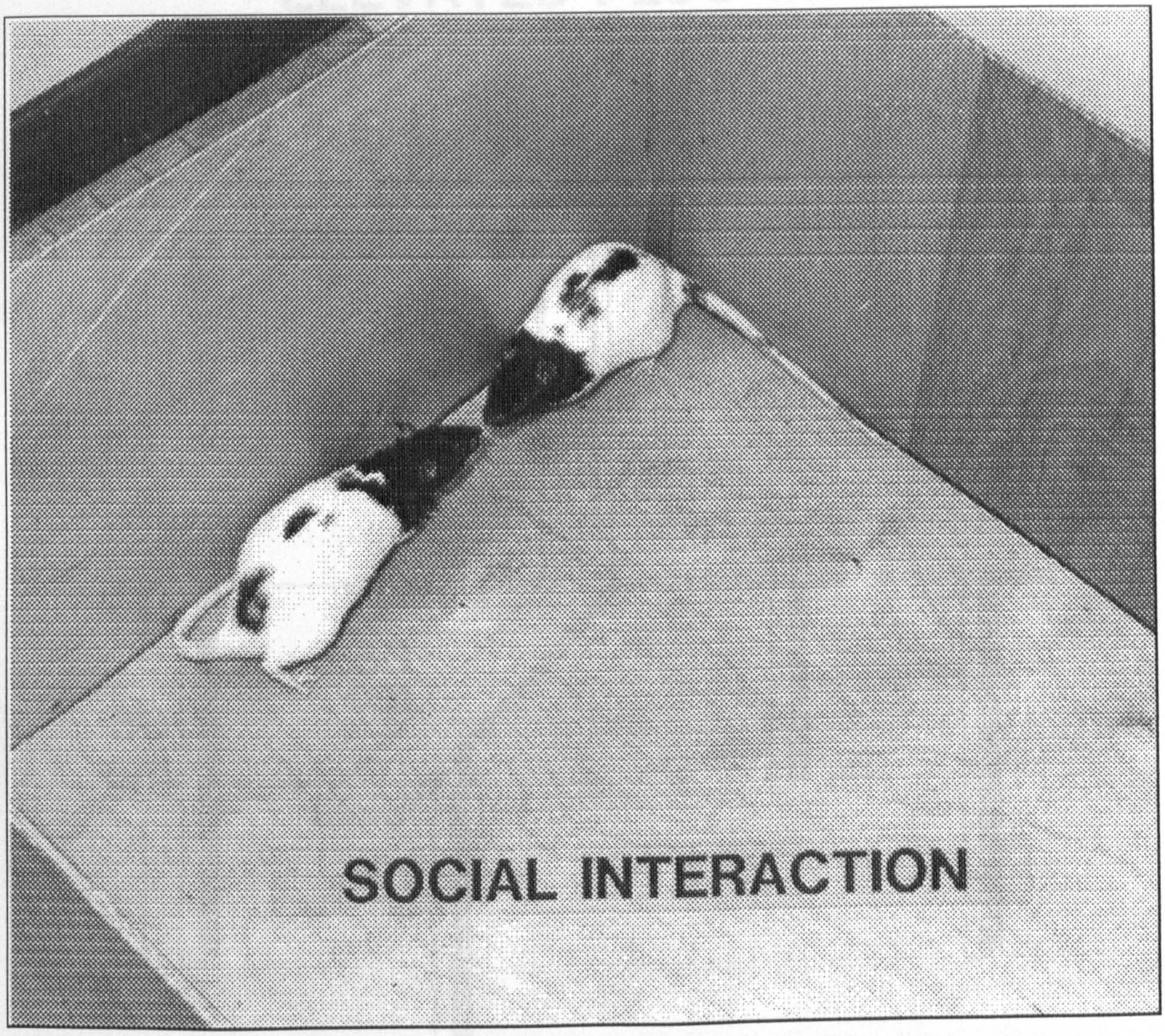

Figure 1.1 The social interaction test arena showing a pair of male hooded Lister rats engaging in social interaction.

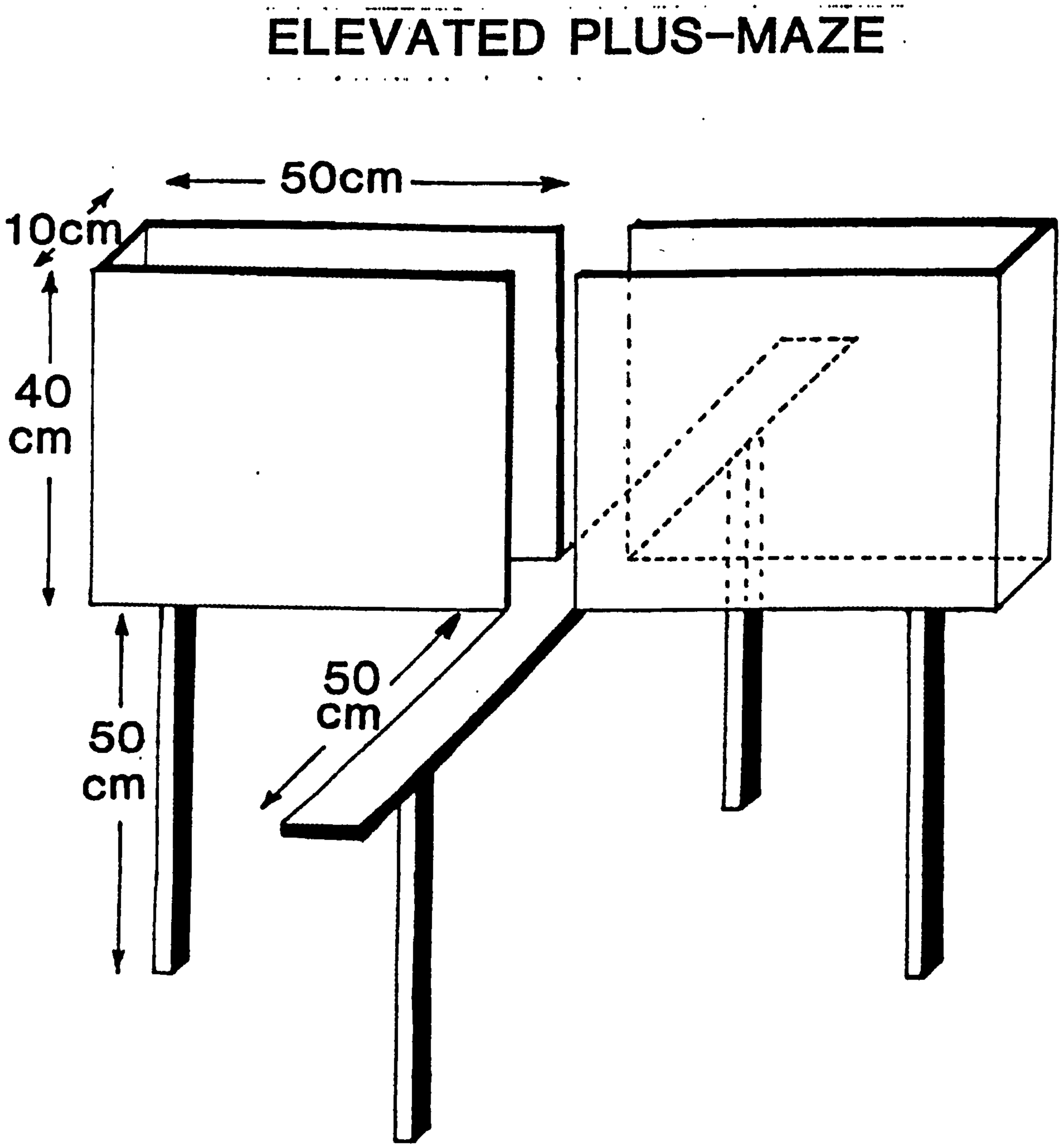

Figure 1.2 Diagrammatic representation of the elevated plus-maze apparatus.

There is increasing evidence to suggest that different animal tests of anxiety measure different types of anxiety (Flaherty et al., 1998; Ramos et al., 1997; Belzung and Le Pape, 1994; File, 1992). Factor analysis studies have shown the factor loadings for the social interaction, elevated plus-maze and Vogel punished drinking tests all load on different factors (Fernandes and File, 1996; File, 1992), thus suggesting that the anxiety generated in these tests is different. There is also evidence to suggest that the anxiety induced on re-exposure to the plus-maze (Trial 2) is different from that in animals that are naive to the plus-maze (Trial 1; Rodgers et al., 1997; File, 1992). Evidence to support this is that benzodiazepines are anxiolytic on Trial 1 but ineffective on Trial 2 (Rodgers and Shepherd, 1993; Rodgers et al., 1992; File, 1990b). This has led to the speculation that Trial 2 of the maze may be a model of phobia (File and Zangrossi, 1993).

1.4 Brain regions and neurotransmitters that are involved in anxiety

There are a number of brain structures that have been implicated in mediating anxiety. These include the raphé nuclei, median (MRN) and dorsal (DRN), the periaqueductal gray (PAG), and limbic structures, such as the dorsal hippocampus, lateral septum and the central and basolateral nuclei of the amygdala (for review see Menard and Treit, 1999). Accumulating evidence from lesioning studies and central drug administration has shown that different brain regions and neurotransmitters control behaviour in different animal tests of anxiety (Menard and Treit, 1999, 1996; Treit and Menard, 1997). There is a great deal of evidence suggesting that benzodiazepine agonists

administered into different brain regions produce reasonably consistent anxiolytic effects in a variety of animal tests. However, evidence regarding the effects of 5-HT_{1A} receptor agonists, 5-HT_2 compounds and 5-HT_3 receptor antagonists is somewhat less consistent, both anatomically and behaviourally.

1.4.1 The GABAergic system

Gamma-aminobutyric acid (GABA) is the major inhibitory transmitter in the brain and thus suppresses neuronal firing thereby inhibiting and regulating other neurotransmitters (Ninan, 1999). GABAergic neurones have been shown to be present in all areas of the central nervous system (CNS), in various densities, and it is estimated that they account for one third of all neurotransmission in the CNS (Shephard, 1987). To date there are three known GABA-receptors, the $GABA_A$, $GABA_B$ and $GABA_C$ receptors (Sigel and Buhr, 1997). $GABA_B$ receptors are G protein-coupled receptors and upon agonist binding they mediate hyperpolarization of post-synaptic membranes and inhibition of neurotransmitter release from presynaptic terminals (Marshall et al, 1999). They have been implicated in numerous neuronal processes (Couve et al., 2000). However, $GABA_A$ receptors are the most characterised of the GABA receptors and are members of the ligand-gated ion channel superfamily (Johnston, 1996). They are pentameric structures that surround a central core through which Cl^- ions pass (Olsen and Tobin, 1990). The receptor is composed of 5 sub-units (α, β, γ, δ and ρ) and several different forms of these subunits have been identified, with different combinations of these subunits conferring marked differences in pharmacology (Mihic et al., 1995; Olsen and Tobin, 1990). When GABA or a $GABA_A$ agonist binds to the receptor the chloride channel opens and allows the intracellular flow of Cl^- ions. This influx inhibits the activity of the neurone to conduct impulses by becoming hyperpolarised. There are binding sites on the $GABA_A$ receptor for other modulatory agents, including the

benzodiazepines, barbiturates, ethanol, steroids and some general anaesthetics (Johnston, 1996).

Benzodiazepine receptors are widely distributed throughout the CNS and are particularly abundant in limbic structures such as the hippocampus, septum and amygdala (Niehoff and Kuhar, 1983; Young and Kuhar, 1980). They are situated on the $GABA_A$ receptor and the benzodiazepines influence the potency of GABA by allosterically modulating the receptor. The benzodiazepine binding site is thought to be situated on the α subunit and the presence of the γ subunit is required of benzodiazepine potentiation of the GABA response (Wu et al., 1994; Olsen and Tobin, 1990). Unlike the barbiturates, which prolong the mean channel opening time of $GABA_A$ receptor, the benzodiazepines increase the frequency of single channel openings (Study and Barker, 1981). Benzodiazepines are unable to open the channel in the absence of GABA.

The full importance of the benzodiazepine binding site was that its activation could result in both anxiolytic and anxiogenic effects. Thus, benzodiazepine agonists (e.g. diazepam, chlordiazepoxide) cause an increase in GABA transmission eliciting an anxiolytic effect (Smith and Olsen, 1995; Zorumski and Isenberg, 1991), whilst benzodiazepine inverse agonists (e.g. FG 7142) cause a decrease in transmission and elicit an anxiogenic effect (Biggio et al., 1987; Stephens et al., 1984; Thiebot et al., 1984). There is a third class of compounds acting at the benzodiazepine receptor, the benzodiazepine antagonists (e.g. flumazenil). These compounds have few, if any,

pharmacological effects on their own but they antagonise the effects of the benzodiazepine agonists and inverse agonists (Haefely, 1983). A recent study has shown that the α2 subunit of the $GABA_A$ receptor may be involved in the anxiety, as mice with this subunit knocked out showed no response to diazepam where as wild-type mice elicited an increase in both the time spent and the number of entries in the open arms (Low et al., 2000). The α1 subunit has been shown to mediate the sedative, amnesic and anticonvulsant actions of diazepam (McKernan et al., 2000; Rudolph et al., 1999).

Direct administration of benzodiazepines into midbrain (DRN and MRN) and limbic structures, has robust anxiolytic effects in tests of both conditioned and unconditioned anxiety (Gonzalez et al., 1998, 1996; Gonzalez and File, 1997; Pesold and Treit, 1996, 1995, 1994; Plaznik et al., 1994; Stefanski et al., 1993; Hodges et al., 1987; Scheel-Kruger and Petersen, 1982; Shibata et al., 1982; Thiebot et al., 1980). Many of these anxiolytic effects were reversed by administration of the benzodiazepine antagonist flumazenil (Gonzalez and File, 1997). Agonist stimulation of the benzodiazepine receptor system does not uniformly modify anxiety. For example, administration of midazolam into the septum increased open arm entries in the elevated plus-maze but did not affect shock-probe avoidance, whereas intra-amygdaloid midazolam dramatically impaired shock-probe avoidance but had no effect on open arm entries (Pesold and Treit, 1994). These findings suggest that distinct sub-populations of benzodiazepine receptors (e.g. those in the septum and the amygdala) may have differing roles in different types of anxiety or that a different GABA tone is generated in different brain regions in different tests of anxiety. Administration of the inverse agonist methyl β-carboline-3-carboxylate (β-CCM) significantly decreased the time rats spent in social interaction after administration into the DRN (Hindley et al., 1985). This effect was blocked by the co-administration of flumazenil.

1.4.2 The Serotonergic System

The serotonergic system has been thought to play an important role in anxiety responses in animals and humans for a number of years (for review see Griebel, 1995). The cell bodies of the 5-HT neurones are found in both the dorsal (DRN) and median (MRN) raphé nuclei, and ascending fibres project from these areas to regions such as hippocampus, septum and amygdala.

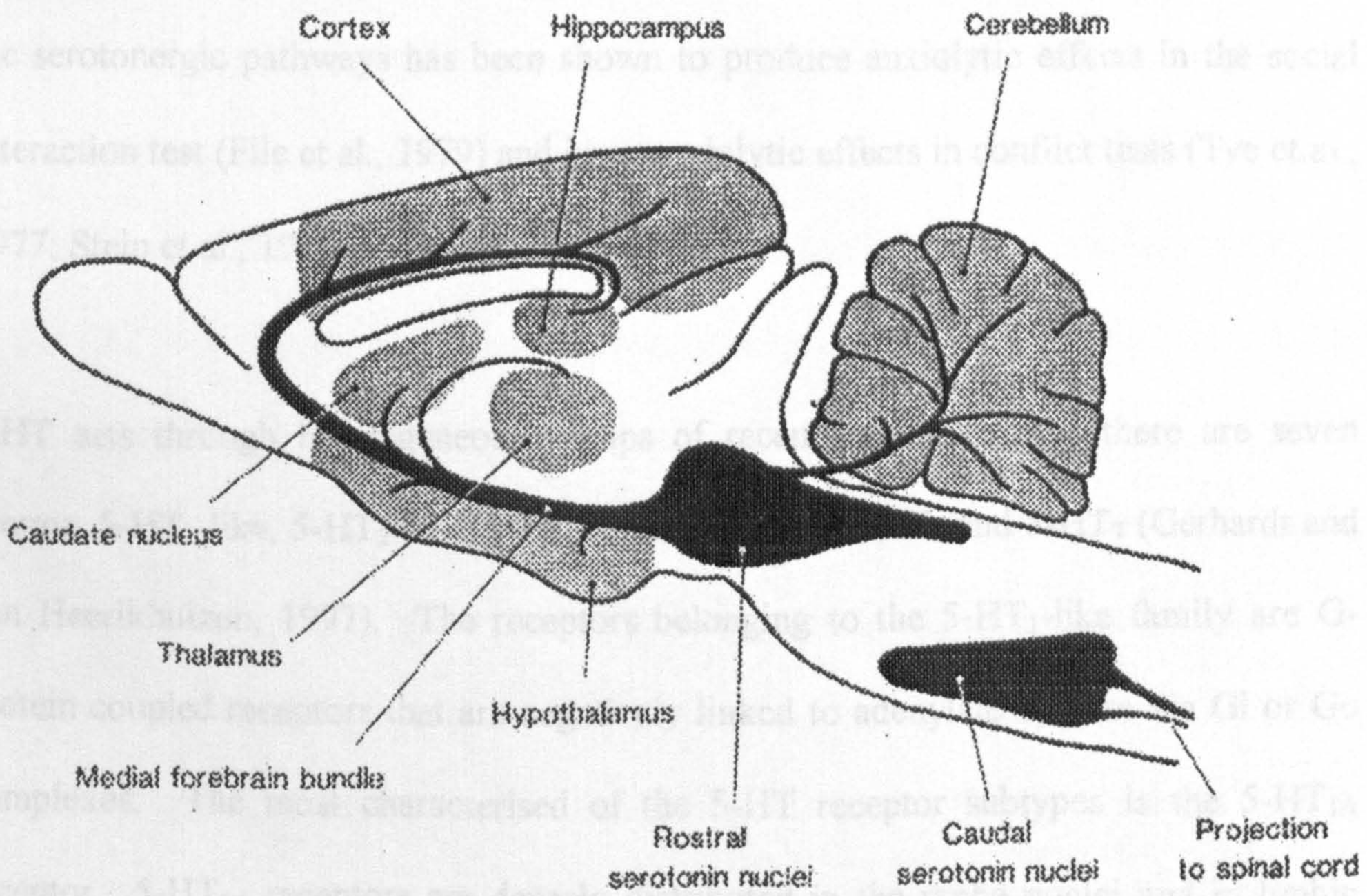

Figure 1.3 Schematic diagram of the serotonergic pathways in the rat brain. The location of the main groups of cell bodies and fibre tracts are shown in black. Grey areas show the location of the serotonergic terminals.

It was suggested, very simplistically, that an increase in 5-HT transmission resulted in increased anxiety, and conversely that a decrease in transmission decreased anxiety (Iversen, 1984). It has been reported that one of the mechanisms of the anxiolytic actions of benzodiazepines is the reduction of 5-HT in the brain (Stein et al., 1977, 1975). Indeed, biochemical and electrophysiological evidence has shown that benzodiazepines and GABA inhibit the synthesis and metabolism of 5-HT (Nishikawa and Scatton, 1986). Furthermore, benzodiazepines and GABA have been shown to have a depressant action on serotonergic activity (Collinge et al., 1983). Lesioning of the serotonergic pathways has been shown to produce anxiolytic effects in the social interaction test (File et al., 1979) and have anxiolytic effects in conflict tests (Tye et.al., 1977; Stein et al., 1975).

5-HT acts through heterogeneous groups of receptors and to date there are seven groups: 5-HT_1-like, 5-HT_2-like, 5-HT_3, 5-HT_4, 5-HT_5, 5-HT_6 and 5-HT_7 (Gerhardt and van Heerikhuizen, 1997). The receptors belonging to the 5-HT_1-like family are G-protein coupled receptors that are negatively linked to adenylate cyclase via Gi or Go complexes. The most characterised of the 5-HT receptor subtypes is the 5-HT_{1A} receptor. 5-HT_{1A} receptors are densely distributed in the raphé nuclei and in limbic areas, such as the hippocampus, septum and amygdala (Gerhardt and van Heerikhuizen, 1997; Waeber and Moskowitz, 1995; Pazos et al., 1985; Pazos and Palacios, 1985). Agonists for the 5-HT_{1A} receptors have been studied in a wide range of animal tests of anxiety but unlike the benzodiazepines, they show inconsistent effects after systemic administration. A possible explanation for these observations is

that stimulation of different 5-HT_{1A} receptors may have different effects on anxiety. The 5-HT_{1A} receptors in the raphé nuclei are situated on the cell bodies or dendrites and act as autoreceptors. Activation of these somatodendritic autoreceptors leads to a reduction in the neuronal firing rate of the 5-HT neurones (Sprouse and Aghajanian, 1987), and thus to a suppression of 5-HT synthesis, 5-HT turnover and 5-HT release in the terminal regions of the limbic system (Bohmaker et al., 1993). The activation of these presynaptic receptors is believed to cause the anxiolytic effects of 5-HT_{1A} agonists. In support of this theory, administration of 5-HT_{1A} receptor agonists, such as 8-OH-DPAT and buspirone, to the MRN and DRN has resulted in anxiolytic effects in the social interaction test (File et al., 1996a; Andrews et al., 1994; Hogg et al., 1994; Higgins et al., 1992, 1988), the elevated plus-maze test (File and Gonzalez, 1996; File et al., 1996a) and in the footshock induced ultrasonic vocalisation (Schreiber and De Vry, 1993). File et al. (1996b) has reversed the anxiolytic effect seen in the social interaction test after co-administration of 8-OH-DPAT with the 5-HT_{1A} receptor antagonist WAY 100,635 thus confirming that the anxiolytic effect was mediated by activation of the 5-HT_{1A} receptors. Furthermore, knockout mice that lack 5-HT_{1A} receptors show increased anxiety compared to wild-type mice (Parks et al., 1998; Heisler et al., 1998). This increased anxiety is thought to be due to the mice lacking 5-HT_{1A} receptors in the DRN and thus having no inhibitory action on the neurone thereby causing an increase in 5-HT in the terminal regions.

In contrast to these results, the administration of 5-HT_{1A} receptor agonists at post-synaptic sites is more complex as administration in to the limbic structures results in

both anxiolytic and anxiogenic effects in animal tests of anxiety. After direct administration of 8-OH-DPAT to the dorsal hippocampus and lateral septum anxiogenic effects have been observed in the social interaction test (Cheeta et al., 2000a; File et al., 1996b; Andrews et al., 1994) and the elevated plus-maze test (Cheeta et al., 2000a; File et al., 1996b). These anxiogenic effects have been show to be receptor specific as they have been reversed by the co-administration of 8-OH-DPAT with WAY 100,635 (Cheeta et al., 2000b; File et al., 1996b). Anxiogenic effects have also been observed in the social interaction test after administration of 8-OH-DPAT to the amygdala, but no effects have been observed in the elevated plus-maze (Gonzalez et al., 1996). In contrast, administration of 5-HT_{1A} agonists to the dorsal hippocampus and septum has induced anxiolytic effects in conflict tests (Przegalinsk et al., 1994; Stefanski et al., 1993). Deakin and Graeff have attempted to reconcile this discrepancy and produce a unifying hypothesis by suggesting that the effects of 5-HT on anxiety may be neuroanatomically specific and dependent on the anxiety in question (Deakin and Graeff, 1991; Graeff et al., 1996). They propose that the panic-like reflexes elicited by exposure to acute unconditioned aversive stimuli arise because of activation of the PAG, and that the anxiolytic effects of 5-HT are mediated by this neuroanatomical substrate (Deakin and Graeff, 1991). This idea is supported by the observation that electrical stimulation of the PAG gives rise to panic-like responses similar to those evoked by unconditioned aversive stimuli in rats (Graeff et al., 1993) and humans (Nashold et al., 1974). Furthermore, enhanced serotonergic transmission within the PAG has been shown to decrease the aversiveness of direct stimulation of this brain structure (for review see Graeff, 1994).

Clinical trials have shown 5-HT_2 receptor antagonists, such as ritanserin, to be important in the treatment of GAD and some phobias (Bersani et al., 1991; Bressa et al., 1987). The 5-HT_2 receptor family consists of the 5-HT_{2A-2C} receptors. These receptors act through Gq proteins and stimulate phosphoinositol hydrolysis. 5-HT_{2A} and 5-HT_{2C} receptors are predominantly located in the basal ganglia and in the limbic system, where as 5-HT_{2B} receptors have a restricted distribution with the greatest expression occurring in the lateral septum and dorsomedial hypothalamus (Menard and Treit, 1999; Griebel, 1995). As is seen for the 5-HT_{1A} agonists, conflicting results have been seen in some animal models of anxiety after systemic administration of drugs acting on the 5-HT_2 receptors (Menard and Treit, 1999; Griebel, 1995).

1.5 The Cholinergic System

The central cholinergic system in the rat projects throughout the brain. It arises within the basal forebrain cholinergic nuclei (medial septum, diagonal band nucleus, nucleus basalis and the substantia inominata) and the pedunculopontine tegmental nucleus. The basal forebrain nuclei innervate the cortex, hippocampus, amygdala and olfactory bulbs. The pedunculopontine tegmental nucleus projects to the basal forebrain cholinergic nuclei, lateral septum, thalamus, raphé nuclei, globus pallidus, locus coeruleus, substantia nigra, interpeduncular nucleus and cerebellum.

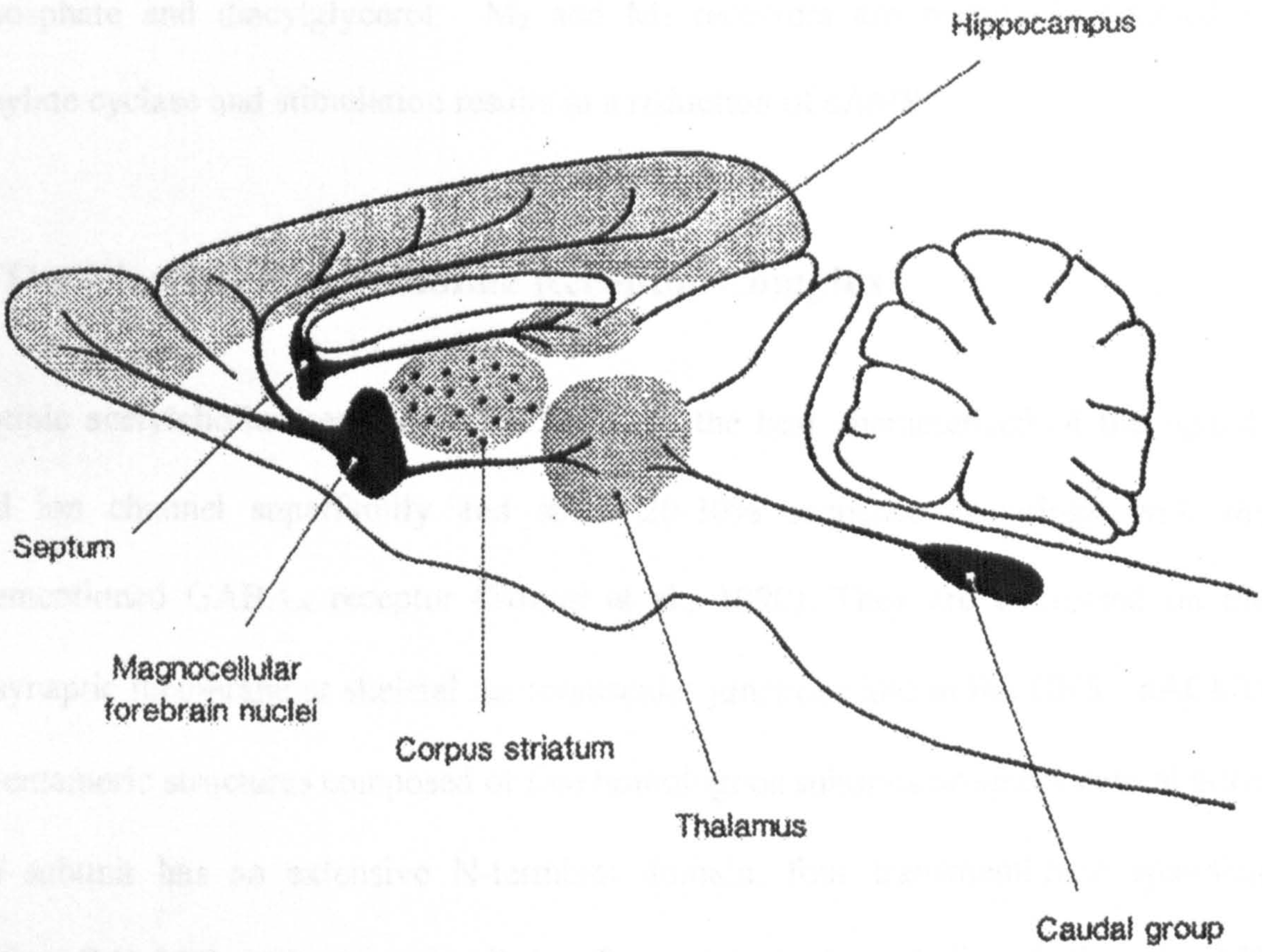

Figure 1.4 Schematic diagram of the cholinergic pathways in the rat brain. The location of the main groups of cell bodies and fibre tracts are shown in black. Grey areas show the location of the cholinergic terminals.

Cholinergic receptors fall into two classes, muscarinic (mAChRs) and nicotinic (nAChRs; discussed in section 1.5) acetylcholine receptors. Both types of receptors bind ACh, but they can be distinguished because there are agonists and antagonists that bind exclusively to one type of ACh receptor or the other. To date there are five muscarinic receptors (M1-M5) that have been cloned (Caulfield and Birdsall, 1998). All of these receptors are linked to GTP-binding protein and have the characteristic 7 transmembrane spanning domains. M_1, M_3 and M_5 receptors are positively coupled to

phospholipase C, and stimulation of these receptors result in an increase in inositol triphosphate and diacylglycerol. M_2 and M_4 receptors are negatively coupled to adenylate cyclase and stimulation results in a reduction of cAMP.

1.6 The Nicotinic Acetylcholine Receptor Complex

Nicotinic acetylcholine receptors (nAChRs) are the best characterised of the ligand-gated ion channel superfamily and share 20-30% sequence homology with the aforementioned $GABA_A$ receptor (Stroud et al., 1990). They are expressed on the postsynaptic membrane at skeletal neuromuscular junctions and in the CNS. nAChRs are pentameric structures composed of five homologous subunits around a central pore. Each subunit has an extensive N-terminal domain, four transmembrane spanning domains (M1-M4) and an extracellular C-terminus. It is believed that the M2 transmembrane domain from each subunit forms the wall of the ion channel, whilst the other three domains form the outer crust (Lukas, 1999). In muscle, the nAChR is composed of five subunits, 2 α and 1 each of β, γ and δ subunits. However, in the CNS the nAChR is composed of only two different subunits, α and β. The α subunit exists in 8 isoforms (α2-9), while the β subunit exists in only 3 isoforms (β2-4). These α and β sub-units can assemble into a wide diversity of combinations and produce receptors with distinct pharmacological and kinetic specificities (Picciotto et al., 2000; Galzi and Changeux, 1995). However, the α7-α9 subunits have been found to form homomeric nAChRs (Picciotto et al., 2000; Galzi and Changeux, 1995).

Within the mammalian CNS two major nAChR groups have been defined using radioligand binding techniques:

- those with a high affinity binding site for (-)-nicotine and are labelled by [^{3}H]-acetylcholine and [^{3}H]-nicotine
- those with a high affinity binding site for α-bungarotoxin (αBgTx)

The branch of the family that binds [^{3}H]-acetylcholine and [^{3}H]-nicotine includes nAChRs composed of α2-α5 and β2 or β4 subunits (Flores et al., 1992; Whiting et al., 1991; Whiting and Lindstrom, 1986). The high affinity (-)-nicotine binding sites of the brain are composed of nAChRs with the subunit stoichiometry $(\alpha)_2(\beta)_3$ (Cooper et al., 1991). The α4β2 subtype is the main nAChR seen in the brain and it binds nicotine with high affinity and is widely distributed (Galzi and Changeux, 1995; see Table 1.3). The branch of the nAChR gene family, which includes nAChRs that can bind αBgTx, is composed of α7, α8 and α9 subunits (Seguela et al., 1993; Schoepfer et al., 1990). All of these subunits, when expressed from their cDNAs, can form homomeric nAChRs, which are characterised by rapid desensitisation and high permeability to Ca^{2+} (Gerzanich et al., 1994). α7 nAChRs are widely distributed in the brain and have an overlapping but distinct distribution pattern compared to the α4β2 subtype. These receptors are rapidly desensitised and are involved in phasic synaptic responses. The α8 subunit has not been found in mammals, and the α9 subunit has only been found in limited areas of rodent brain and cochlea (Elgoyhen et al., 1994).

Table 1.3 Distribution of nAChR subunits in the rat brain.

Subunit	Brain area with high distribution	References
α3	Hippocampus Thalamus Substantia nigra	Wada et al., 1989; Goldman et al., 1986
α4	Hippocampus Dorsal raphé nucleus Hypothalamus Ventral tegmental area Substantia nigra Thalamus Locus coeruleus	Lobron et al., 1995; Bitner et al., 2000; Alkondon et al., 1994; Goldman et al., 1987; Léna et al., 1999
α6	Ventral tegmental area Substantia nigra Thalamus	Léna et al., 1999; Le Novere et al., 1996
α7	Hippocampus Amygdala Cortex Ventral tegmental area	Zarei et al., 1999; Seguela et al., 1993; Davies et al., 1999
β2	Thalamus Hypothalamus Ventral tegmental area Substantia nigra Hippocampus Locus coeruleus	Dominguez del Toro et al., 1994; Zarei et al., 1999; Léna et al. 1999; Hill et al., 1993; Deneris et al., 1989
β4	Dentate gyrus Locus coeruleus	Dineley-Miller and Patrick, 1992

1.7 Nicotinic Acetylcholine Receptor Ligands

Nicotine has been the main nicotinic receptor agonist that has been used to study nAChR function. Nicotine is a tertiary amine consisting of a pyridine ring and a pyrolidine ring and it can exist in two forms; the (S)-nicotine isomer is the active form which binds to nAChRs and is found in tobacco, and (R)-nicotine which is a weak agonist at nAChRs and is found in only small quantities in tobacco (Royal College of Physicians, 2000). However, there are now a number of ligands available which selectively modulate the nAChR, such as ABT-418 (S)-3-methyl-5-(1-methyl-2-pyrrolidinyl)isoxazole, cytisine, 1,1-dimethyl-4-piperazinium (DMPP) and epibatidine;. It has been shown that the sensitivity of the nAChRs to these nicotinic agonists depends on the subunit combination (see Table 1.4). Thus, the determination of potency rank order among nicotinic agonists is a powerful tool to distinguish nAChRs in native cells. Although small differences in affinity could be ascribed to different α subunits, the major factor in determining agonist affinity appears to be the nature of the β subunit. β2-containing nAChRs possess consistently higher affinities for ACh, cytisine, DMPP, and nicotine than do β4-containing nAChRs (Picciotto et al., 2000).

In addition to these nAChR agonists there are also a number of competitive and non-competitive antagonists to the nicotinic receptor that have been identified. The competitive antagonists, such as methylcaconitine (MLA), αBgTx and dihrdro-β-erythroidine (DHβE), compete with agonists for binding to the same overlapping sites

(for review, see Gotti et al., 1997). Like for the agonists, the potency of these antagonists at the nAChR is dependent on the subunit composition. Both MLA and αBgTx preferentially bind to α7-type nAChRs, where as DHβE blocks α4β2 nAChRs. The non-competitive antagonists, such as mecamylamine and chlorisondamine, do not bind to the agonist binding site. They act by occluding the ion channel of the nAChR.

Table 1.4 Functional nAChR subtypes present in the rat brain with relative agonist potencies at each subtype. ABT-418 ((S)-3-methyl-5-(1-methyl-2-pyrrolidinyl)isoxazole); ACh, acetylcholine; Cyt, cytisine; DMPP. 1,1-dimethyl-4-piperazinium; Epi, epibatidine; Nic, nicotine. Modified from Royal College of Physicians (2000).

Sub-unit Combination	Rank order of potency
α2β2	Nic > DMPP > ACh > Cyt
α3β2	DMPP = ACh ≥ Nic > Cyt
α4β2	Epi > Nic = ACh > DMPP > Cyt
α2β4	Cyt > Nic > ACh > DMPP
α3β4	Cyt > Nic = ACh = DMPP
α4β4	Cyt > Nic > ACh > DMPP
α7	Nic > Cyt > DMPP > ACh

1.8 Allosteric Regulation of the Nicotinic Acetylcholine Receptor

The nAChR is controlled allosterically by a ligand (e.g. acetylcholine or nicotine) binding to sites located on two of the subunits. On heteromeric nAChRs, the agonist binding site is thought to be at the interface between the α subunit and the adjacent β subunit (Galzi and Changeux, 1995). However, this is not the case for the α5 receptor subunit as it is incapable of binding an agonist because it lacks a tyrosine residue that is essential for binding (Conroy et al., 1992). The homomeric nAChRs have five agonist binding sites located at the junction between neighbouring subunits. For both the heteromeric and homomeric nAChRs, binding of ligand at one of the binding sites increases the affinity of the second site for the ligand (Léna and Changeux, 1993). Activation of the nAChR results in opening of the associated central channel, which allows entry of Na^+, K^+ and Ca^{2+} (Cooper et al., 1991; Wonnacott, 1990). However, the channel only remains open for a short time before it undergoes a series of conformational changes that cause the receptor to become desensitised. There are several lines of experimental evidence to suggest that the nAChR may exist in a minimum of 4 interconvertible states, the resting (R), active (A), intermediate (I) and desensitised (D) states (Figure 1.3). In the active state (A), the receptor has low affinity for nicotinic agonists but in the two desensitised closed channel states, I and D, the receptor has enhanced affinity for these agonists. However, only the active state of the receptor is functionally active.

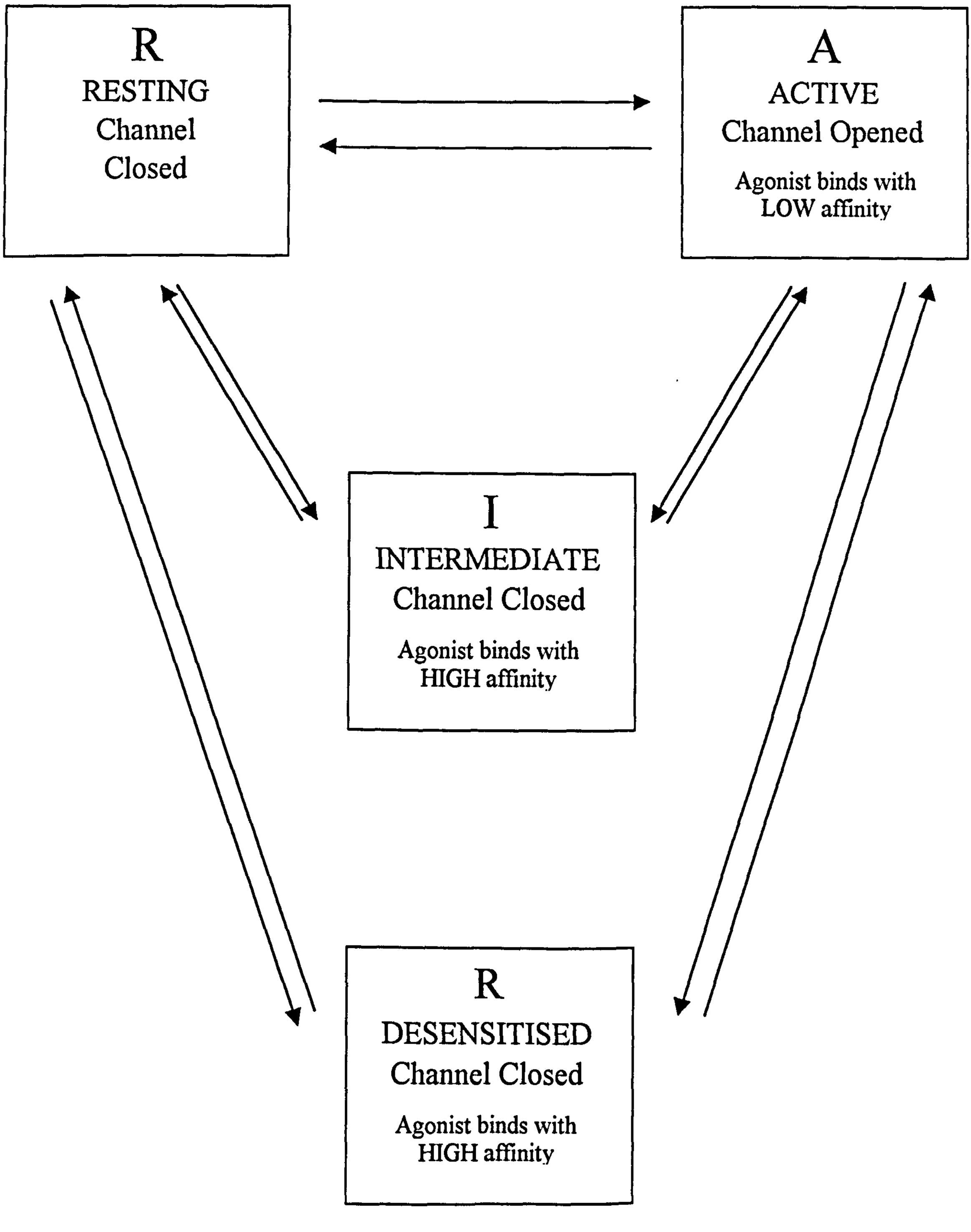

Figure 1.5 Proposed conformational states of the nicotine acetylcholine receptor. Modified from Galzi and Changeux, 1995.

The rate at which the nicotinic receptor proceeds through the various conformational states depends on many factors, including the subunit composition. The α7 and α8 homomeric receptors desensitise very rapidly, whereas the heteromeric receptors undergo activation and desensitisation transitions with kinetics that vary with the α and β subunit composition. The nAChRs composed α4β2 subunits, for which nicotine has a high affinity, desensitise much more rapidly when exposed to nicotine than do receptors made up of α3β4, for which nicotine has a low affinity. Thus, the higher the affinity of a specific agonist for a particular nAChR subtype, the faster the agonist activates and deactivates the receptor.

Contrary to dogma that chronic agonist exposure leads to downregulation of its receptors, nicotine exposure induces increases in number ("upregulation") of CNS radioligand binding sites in both humans and animals. Post-mortem brains of smokers have an increased number of nicotinic receptors compared to non-smokers in a number of brain regions (Perry et al., 1999; Breese et al., 1997; Benwell et al., 1988). Continuous infusion and intermittent administration of nicotine treatment has been shown to increase the number of putative nAChR in the brain of both rats and mice (Collins et al., 1994, 1990, 1988; Ksir et al., 1987; Marks et al., 1985, 1983). This increased receptor density is very widespread in rodent brain, with more than two-thirds of the brain areas examined by autoradiography showing this effect after chronic nicotine treatment (Marks et al., 1992; Kellar et al., 1989). The extent of the upregulation of these receptors varies considerably between brain regions, with increases of 60-100% in the cortex and hippocampus, whereas in other brain regions

there is no significant increases (Flores et al, 1997; Marks et al., 1992; Kellar et al., 1989).

In addition to this upregulation of receptors, chronic nicotine treatment can be associated with a long-lasting downregulation in receptor function (Hsu et al., 1996; Peng et al., 1994; Marks et al., 1993a, b). This decrease in function is thought to be due, at least in part, to receptor desensitisation. It is this long-lasting receptor desensitisation that is thought to induce the receptor upregulation (Schwartz and Kellar, 1985; Marks et al., 1993a, b).

1.9 Functional Role of Nicotinic Acetylcholine Receptors

Many of the neuronal nAChRs, unlike the postsynaptic nAChRs of muscle, are predominantly found on the presynaptic terminals (Wonnacott, 1997). They thus influence neuronal activity by altering the firing rate and/or pattern of the neurone via activation of somatodendritic receptors, and by activating the receptors on the nerve terminals they control neurotransmitter release. There is evidence that both of these mechanisms are implicated in the effects of nicotine in the brain.

Presynaptic nAChRs in the brain modulate the release of several neurotransmitters (see Vizi and Lendvai, 1999; Wonnacott, 1997, for recent reviews). Nicotine and nicotinic agonists have been shown to increase the release of ACh, dopamine, noradrenaline (NA), 5-HT and GABA from synaptosomal and tissue slice preparations, and in whole animals using microdialysis (see Table 1.5). The rewarding effects of nicotine are

thought to be due to stimulation of dopamine release and thus there has been extensive research on the release of this transmitter. In the rat striatum nicotine, DMPP, epibatidine and cytisine have all been shown to increase dopamine release using synaptasomes (Grady et al., 1997; Clarke and Reuben, 1996; Rowell and Hillebrand, 1994), in vitro slices (Sacaan et al., 1996) and in vivo (Dajas-Bailador et al., 1998).

The dorsal hippocampus is a structure that has been shown to be crucial in mediating anxiety. A number of studies in rodents have shown that stimulation of presynaptic nAChRs in this area cause release of a number of neurotransmitters, including ACh, dopamine, GABA and NA (see Table 1.5). For example, the nicotinic agonists DMPP and epibatidine have also been shown to enhance the release of NA from the hippocampus and whole hippocampal slices in a $[Ca^{2+}]_o$-dependent and TTX-sensitive manner (Sershen et al., 1997). The effect of nicotine on 5-HT release has been studied less extensively. Lendvai et al. (1996) have shown DMPP and lobeline can increase 5-HT release from the hippocampus, but nicotine, cytisine, and epibatidine have no effect. However, a recent study by Kenny et al. (2000a) showed that nicotine (50-500μM) caused a concentration-dependent increase in 5-HT release, that was antagonised by mecamylamine indicating the involvement of nicotinic receptors.

Table 1.5 Neurotransmitters that are modulated by presynaptic nicotinic receptors within various rat brain structures. ACh, acetylcholine; 5-HT, 5-hydroxytryptamine; GABA, γ-aminobutyric acid; DRN, dorsal raphé nucleus.

Transmitter	Brain Region	Reference
ACh	Hippocampus Cortex Striatum	Wilkie et al., 1996; Toide and Arima, 1989; Araujo et al., 1988
Dopamine	Hippocampus Striatum	Kaiser et al., 1998; Toth et al., 1992
5-HT	Hippocampus DRN	Kenny et al., 2000a; Li et al., 1998; Lendvai et al., 1996
GABA	Hippocampus Septum	Alkondon et al., 1999, 1997; Yang et al., 1996
Noradrenaline	Hippocampus	Kiss et al., 1997; Sershen et al., 1997; Clarke and Reuben, 1996; Sacaan et al., 1996

1.10 Nicotine and Behaviour

Nicotine has a complex neuropharmacological profile and it influences several neurotransmitters and thus produces a range of physiological and behavioural effects in both laboratory animals and humans (Stolerman et al., 1995). These effects range from changes in body temperature, locomotor activity and reward, to enhancement of cognitive function and attention. These effects can be influenced by a number of factors including dose, route of administration, species and strain of animal.

1.10.1 Nicotine and Reward

Nicotine, like other drugs of addiction such as the psychomotor stimulants, depressants and opiate narcotics, is thought to express its rewarding effects by stimulating the

mesolimbic dopamine system (Pontieri et al., 1996; Schulteis & Koob, 1994; Koob, 1992; Wise and Bozarth, 1987). Acute nicotine administration potently stimulates dopamine release from the nucleus accumbens (Nacc), particularly from within the shell region. This effect on NAcc dopamine release is blocked by intra-ventral tegmental area (VTA) injection of the nicotinic receptor antagonist, mecamylamine, but not by NAcc mecamylamine injection (Nisell et al., 1994). This suggests that the nicotinic receptors located within the VTA, and not within the NAcc, predominately mediate the effect of nicotine on dopamine release in vivo.

The ability of nicotine to support self-administration in rats under a limited access schedule (Corrigall & Coen, 1989) and to condition a place preference (Risinger & Oakes, 1995; Shoaib et al., 1994) are thought to reflect the 'rewarding' properties of nicotine produced by it's ability to evoke NAcc dopamine release. Accordingly, Picciotto et al (1997) have recently demonstrated that nicotine can no longer elicit mesolimbic dopamine release in mice lacking the β2 subunit and that this decrease in dopamine release is accompanied by a dramatic decrease in nicotine self-administration in these mutant mice. Furthermore, lesioning of the mesolimbic dopamine neurones significantly attenuates nicotine self-administration in rats (Corrigall et al., 1992). Recent data suggests that dopamine release in the NAcc may not mediate the rewarding effects of nicotine *per se*, but may be more related to signalling in the brain that a rewarding effect is about to take place. For example, neutral stimuli which by themselves have no rewarding value, have been shown to elicit NAcc release after they have been associated with rewarding stimuli (Phillips et

al., 1993; Damsma et al., 1992). Further, novel stimuli (Rebec, 1998; Ljungberg et al., 1992), stress and aversive stimuli (McCulloch et al., 1993; Young et al., 1993) and stimuli which have been temporally associated but are not rewarding nor aversive (Young et al., 1995) have also been shown to increase NAcc dopamine release. However, Di Chiara has noted that non-rewarding stimuli, as described above, preferentially stimulate dopamine release within the core of the nucleus accumbens, whereas rewarding stimuli and drugs of abuse preferentially stimulate dopamine release in the shell (see Di Chiara, 2000). Nevertheless, these observations have led Joseph et al. (1996) to speculate that NAcc dopamine release may be involved in attributing salience to a particular stimuli rather than mediating a rewarding effect of that stimuli. Accordingly, although accumbal dopamine release has recently been shown to be involved in acquiring intracranial self-stimulation (ICSS) behaviour once established, there was no change in accumbal dopamine release during ICSS behaviour (Garris et al., 1999). Therefore, it appears that dopamine plays a complex role in mediating reward and that neurotransmitters other than dopamine are also likely to be involved in mediating the effects of nicotine. One possible candidate is 5-HT, which has been suggested to play a role in maintaining cocaine self-administration in mutant mice lacking the dopamine-transporter (Rocha et al., 1998).

1.10.2 Nicotine and Cognition

There is substantial evidence to suggest that the cholinergic system plays an important role in cognition, in both humans and animals (Rezvani and Levin, 2001; Mancuso et al., 1999; Rusted et al., 1998; Warburton and Mancuso, 1998; Levin and Simon, 1998; Levin, 1992), particularly those involving attentional processes. Indeed, marked impairment of cognitive skills can be seen after the disruption of cholinergic neurotransmission in the brain, either by neurodegeneration or pharmacological intervention. The development of conditions such as Alzheimer's disease, in which cognitive impairment is the principal symptom, is thought to be due to degeneration of the cholinergic system. A significant loss in the density of high affinity [^{3}H]-nicotine binding sites is seen in post-mortem brains of Alzheimer disease patients (Newhouse et al., 1997). Nicotine administration via skin patches or injection has been shown to significantly improve attention (White and Levin, 1999; Jones et al., 1992a), learning (White and Levin, 1999) and memory (Parks et al., 1996; Newhouse et al., 1988) in patients with Alzheimer's disease. Disruption of the cholinergic system with muscarinic or nicotinic antagonists, such as atropine or mecamylamine, has shown memory deficits on a number of behavioural tests (Levin et al., 1997; Levin, 1992).

The effects of nicotine on cognition have been studied in animals using a number of tests of learning and memory. Acute treatment with nicotine has been found to improve working memory function in the radial-arm maze (Levin et al., 1998, 1997; Decker et al., 1995), in passive avoidance (Zarrindast et al., 1996; Decker et al., 1994; Brioni and Arneric, 1993), Morris water maze (Socci et al., 1995) and delayed-match-

to-sample tasks (Buccafusco et al., 1996, 1995; Buccafusco and Jackson, 1991; Elrod et al., 1988). Interestingly, after chronic treatment with nicotine no tolerance is seen to the memory improvement effects, and in fact the improvement appears to become more robust (Levin et al., 1993, 1990). Mecamylamine, the non-competitive nAChR antagonist, impairs cognitive function in normal animals and blocks the improvements seen in cognitive performance after both acute (Levin et al., 1997) and chronic (Levin et al., 1999) nicotine treatment.

Nicotinic agonists, such as ABT-418 and epibatidine, that are thought to potently act at the $\alpha4\beta2$ subtype, have been shown to significantly improve cognitive performance in rodents (Decker et al., 1994). ABT-418 has also been shown to significantly enhance delayed-match-to-sample performance in monkeys (Buccafusco et al., 1996). The $\alpha7$ agonist, GTS-21, has also been shown to improve performance in a variety of tests (Arendash et al., 1995). Thus, these results suggest that both the $\alpha4\beta2$ and $\alpha7$ subtypes may be important in mediating the effects of nicotine on cognition.

Recent studies have investigated particular neuroanatomical sites where nicotine and nicotinic agonists may be mediating their effects. The hippocampus has been known for a long time to be involved in memory and attention (Jarrard, 1995) and it is known to contain a number of nAChRs. Indeed, local administration of mecamylamine directly into the hippocampus caused a significant memory deficit (Ohno et al., 1993). Furthermore, administration of DHβE, the $\alpha4\beta2$-selective antagonist, or MLA, the $\alpha7$-selective antagonist, causes significant memory impairment after infusion in to the

hippocampus. Therefore giving further evidence of the involvement of the α4β2 and α7 subtypes in mediating the effects of nicotine on cognition.

1.10.3 Nicotine and Anxiety

There is growing evidence suggesting that the cholinergic system is involved in the modulation of anxiety. It is often reported by smokers that smoking reduces anxiety and tension (Ikard, 1969) and smoking tends to increase during episodes of stress and depression (Breslau et al., 1991; Hughes et al., 1986). However, not all smokers experience anxiolytic effects from smoking (Parrott and Garnham, 1998), and Newhouse et al. (1990) found nicotine increased anxiety in non-smokers. Netter et al. (1998) found that smokers with high neuroticism scores became more anxious and tense after smoking a cigarette. There is general agreement that there is increased anxiety during nicotine withdrawal in smokers (West and Russell, 1985; Shiffman and Jarvik, 1976) and those using nicotine gum (Parrott et al., 1996; Hughes et al., 1990; Keenan et al., 1989). Nicotine patches and tablets have been shown to reduce anxiety in smokers (Netter et al., 1998; Warburton and Mancuso, 1998; Wesnes and Warburton, 1983), which may reflect a reversal of withdrawal anxiety.

In animal studies, nicotine and nicotinic agonists have anxiolytic effects after acute systemic administration in the elevated plus-maze test (Brioni et al., 1993, 1994), the mirrored chamber test (Cao et al., 1993), the light-dark exploration test (Costall et al., 1989b) and the potentiated startle test (Vale and Green, 1986). In contrast to Brioni's report of anxiolytic effects after acute systemic administration in the elevated plus

maze (Brioni et al., 1993, 1994), anxiogenic effects have been reported in this test (Ouagazzal et al., 1999a). In this test the direction of nicotine's effects is not dose-related, since the dose (0.3 mg/kg) that was reported to be anxiolytic by Brioni et al. (1994) fell in the dose-range found to be anxiogenic by Ouagazzal et al. (1999a), and Benwell et al. (1994) found 0.4 mg/kg to be ineffective. Lower doses (0.001-0.1 mg/kg) were shown to be ineffective by Ouagazzal et al. (1999a). These differences in response to an acute injection of nicotine could be due to strain differences and/or differences in the baseline scores. Aversive effects of nicotine have also been reported in operant tests of punished behaviour, and nicotine has been reported to both suppress punished responding (Morrison, 1969), and to function as a punishing stimulus (Takada et al., 1992).

In the social interaction test, it has been shown that acute systemic nicotine produces both anxiolytic and anxiogenic effects that are dose and test-condition dependent (File et al., 1998). In the social interaction test, nicotine has been shown to be dose-dependent, with low doses (0.01 and 0.1 mg/kg) having an anxiolytic effect after acute systemic administration and high doses (0.5 and 1.0 mg/kg) an anxiogenic effect, when animals were tested 30 min after an i.p. injection (File et al., 1998). This study also showed that the effects on anxiety were also dependent on the test condition, with effects only being observed in conditions of moderate anxiety (HF: high light familiar or LU: low light unfamiliar). Similarly, using the technique of self-administration, the dose and test condition determines whether the maintenance of lever pressing behaviour results in, or prevents, nicotine infusions (Goldberg et al., 1983).

As mentioned before, the dorsal hippocampus is a structure that is crucially involved in anxiety disorders. Administration of nicotine bilaterally into the dorsal hippocampus has been shown to induce anxiogenic effects in the social interaction test (File et al., 1998). These effects have also been shown to be dependent on the test condition and as before are only seen in conditions of moderate anxiety (HF, 0.1-8μg; LU, 8μg). The 5-HT_{1A} receptors were implicated in this action, because co-administration into the dorsal hippocampus of the 5-HT_{1A} receptor antagonist, WAY 100,635, reversed the anxiogenic effect of nicotine (Kenny et al., 2000b). Furthermore, it has been shown that nicotine increases 5-HT release in the dorsal hippocampus (Kenny et al., 2000a), thus, suggesting that nicotine exerts its anxiogenic effect by stimulating 5-HT release in this region and activating 5-HT_{1A} receptors. Intra-hippocampal injections of nicotine (0.1-8μg) were ineffective on Trial 1 but on Trial 2 an anxiolytic effect was observed (Ouagazzal et al., 1999a).

The lateral septum has also been identified as an important neuroanatomical substrate mediating nicotine's anxiogenic effects. Anxiogenic effects have been observed in both the social interaction (1-8μg; Ouagazzal et al, 1999b; Cheeta et al., 2000b) and elevated plus maze (1 and 4μg; Ouagazzal et al, 1999b) tests after direct administration of nicotine to this structure. These anxiogenic effects were also antagonised by co-administration of the specific 5-HT_{1A} receptor antagonist WAY 100,635 (Cheeta et al, 2000b), suggesting an important role for 5-HT_{1A} receptors in this brain area in mediating nicotine's anxiogenic effects.

1.11 Tolerance Mechanisms and Withdrawal

The development of behavioural tolerance following the repeated administration results in a reduced response to a given dose of drug, or the need to increase the dosage to maintain the same original effects (Jaffe, 1990; Nestler, 1992). There is thought to be two different types of tolerance. The first is *dispositional tolerance* (pharmacokinetic) which results from changes in the absorption, distribution or metabolism of a drug and might lead to a reduction in the intensity and duration of contact between a given drug and the tissue on which it exerts its characteristic action. The second is *functional tolerance* (pharmacodynamic) which is usually taken to be tolerance that is mediated by changes in the sensitivity of the neuronal, receptor or neurochemical system which may limit a drug's actions (Goudie, 1989). Littleton and Little (1989) have suggested that there may be two distinct models of functional tolerance whereby adaptive changes are recruited to in an attempt to maintain homeostatic balance, decremental and oppositional. An example of decremental tolerance is when the receptor number or the properties of the receptor are changed. With this form of tolerance there is not an obvious indication that the drug is not present. Oppositional tolerance suggests that continued drug treatment results in an oppositional process that comes into effect to counteract the effect of the drug. This oppositional effect could explain the withdrawal syndrome that is often seen when drug treatment is discontinued. There is now evidence to suggest that learning may play an important part in drug tolerance (Young and Goudie, 1995; Goudie and Griffiths, 1996). It is thought that classically conditioned processes may occur following the repeated administration of a drug when it is paired with a known stimulus, such as an environmental cue. These stimuli may

induce compensatory conditional responses, which become paired with the drug and cause a reduction or oppose the direct effect of the drug leading to the development of tolerance. Therefore, tolerance to the behavioural effects of the drug is greater when measured in the environment associated with drug treatment, i.e. the tolerance is context or situation-specific.

Withdrawal refers to the syndrome that emerges following the discontinuation of drug treatment. There are though to be at least three distinct discontinuation syndromes that occur after drug treatment is terminated and patients may have any combination of these: 1) recurrence, when the symptoms that were seen before drug treatment occur, 2) rebound, when the symptoms that occurred before treatment return but with a greater intensity and 3) withdrawal, when symptoms are seen that were not seen before drug treatment (Greenblatt et al., 1990). These syndromes are able to be distinguished as they have different rates of onset and the intensity of the syndromes is varied.

1.11.1 Tolerance to Nicotine's Behavioural Effects

One view of the onset of regular smoking suggests that repeated exposure to nicotine gradually leads to reduced magnitude of its effects, i.e. chronic tolerance. Chronic tolerance to nicotine in smokers leads to a greater effort by the smoker to continue to obtain the same magnitude of reinforcing effects of nicotine and thus is important in nicotine dependence. Repeated exposures to nicotine during the course of a single day may also lead to reduced responding across these exposures, a change that may reflect acute tolerance. Chronic tolerance and acute tolerance in smokers and non-smokers

has been demonstrated for many of nicotine's effects, including subjective and cardiovascular effects (Fattinger et al., 1997; Perkins et al., 1994, 1993, 1989; Arcavi et al., 1994; Russell et al., 1990).

Initial exposure to cigarettes is typically reported as aversive, and many studies have shown that have shown that smokers respond less to the dysphoric effects of nicotine (e.g. tense, dizzy, jittery, light-headed) than non-smokers, thus demonstrating chronic tolerance (Perkins et al., 1994, 1993). Heishman and Henningfield (2000) have shown that non-smokers treated with nicotine for 8 days show tolerance to some of these aversive effects of nicotine and to the sedative effects of nicotine. There is also evidence for acute tolerance to some of these subjective effects of nicotine (e.g. dizzy, light-headed) in both smokers and non-smokers suggesting that novice smokers may be able to adapt rapidly to some of the initial aversive effects of nicotine during a smoking episode, and thus leading to increased ability to smoke more cigarettes per episode.

Nicotine produces a wide range of acute dose-dependent cardiovascular effects in smokers, the most common effects being increased heart rate and blood pressure. Acute (Fattinger et al., 1997; Arcavi et al., 1994; Perkins et al., 1994, 1991) and chronic (Perkins et al., 1994, 1989) tolerance has been shown to both of these cardiovascular effects.

Animal research has shown that there are adaptions to many behavioural and physiological effects of nicotine after chronic treatment with nicotine. The most

studied is nicotine's effect on locomotor activity. An acute injection of nicotine produces a biphasic effect on locomotor activity in rats. After an acute injection of nicotine there is an initial decrease in locomotor activity and approximately 40-60 min after injection a stimulant effect is observed. With repeated nicotine injections, tolerance develops to the initial depressant effects (Stolerman et al., 1995, 1974, 1973; Ksir, 1994; Benwell and Balfour, 1992). The increase in locomotor activity following repeated nicotine injections, which is similar to the increase in activity following other psychostimulants, such as amphetamine and cocaine, has been referred to by many investigators as locomotor sensitisation. There is much evidence to show that tolerance does not develop to this stimulant effect and is in fact enhanced (Shoaib et al, 1997; Ksir et al, 1987). The expression of locomotor sensitisation is blocked by mecamylamine or DHβE (Stolerman et al., 1997; Benwell and Balfour, 1992; Clarke and Kumar, 1983a, b), indicating the involvement of nicotinic receptors.

A number of studies have shown that tolerance can develop very rapidly to the effects of nicotine on antinociception (McCallum et al., 2000, 1999; Wewers et al., 1999). Wewers et al. (1999) demonstrated that male and female rats treated with nicotine (0.3 mg/kg) displayed significantly greater antinociceptive responses, as evidenced by prolonged hot-plate latency during the initial phases of the protocol. However, by day 7 of nicotine treatment the antinociceptive response had disappeared, suggesting tolerance to nicotine (Wewers et al., 1999). McCallum et al. (2000) showed that in rats administered a short (once-daily for 6 days; 0.35 mg/kg) or long (twice-daily for 11 days; 0.66 mg/kg) series of injections tolerance to nicotine-induced antinociception.

Pairings of mecamylamine (1 mg/kg) with nicotine (0.35 mg/kg) for both these treatment regimens blocked the development of tolerance, indicating nicotinic receptor activation was necessary for tolerance to occur.

Acute nicotine causes a decrease in milk intake, but after 6 days of treatment (0.66 mg/kg) with this dose of nicotine tolerance is seen to this effect (McCallum et al., 1999). Mecamylamine blocked tolerance to the effects of nicotine Six daily pairings of mecamylamine (1 mg/kg) with nicotine blocked the development of tolerance to the ability of nicotine to suppress milk intake (0.66 mg/kg). Thus, again indicating the involvement of nicotinic receptors.

A limited number of studies have investigated the development of tolerance to the discriminative stimulus effects of nicotine. It has been shown that acute tolerance can develop to the nicotine discrimination following single boluses of nicotine (0.8 mg/kg) injected 90 min apart (James et al., 1994) or after several days of nicotine treatment (Schechter and Rosecrans, 1972). However, Shoaib et al. (1997) did not show tolerance to the discriminative stimulus of nicotine after once or thrice daily injections of nicotine or continuous infusion for 7 days. These contradictory results suggest that chronic tolerance to nicotine's discriminative stimulus does not develop readily.

With regards to tolerance to anxiety the literature at present is very limited. Costall et al. (1989b) has shown that after 3, 7 and 14 days of chronic nicotine (0.1 mg/kg, i.p.)

treatment tolerance did not develop to the acute anxiolytic effect seen in the light-dark exploration test.

1.11.2 Nicotine Withdrawal

In humans, withdrawal from chronic nicotine results in an abstinence syndrome that is characterised by a number of different symptoms. The most prominent of these symptoms being anxiety, irritability, restlessness, lack of concentration, light-headedness, insomnia, increased hunger and weight gain (Hughes et al., 1991). It is these withdrawal symptoms that are thought to be the cause of the high relapse rate observed during the first few days of smoking cessation (Hildebrand et al., 1997; Hughes et al., 1992). Nicotine patches have been shown to reduce anxiety in smokers (Netter et al, 1998; Warburton and Mancuso, 1998; Wesnes and Warburton, 1983), which may reflect a reversal of withdrawal.

Animal models are an important tool for understanding the neurobiological mechanisms underlying nicotine abstinence. Malin et al. (1992) has described an abstinence syndrome in rats that is characterised by teeth chattering/chews, writhes/gasps, ptosis, tremors/shakes and yawns. These characteristics were seen after 7 days of subcutaneous infusion of 3 or 9 mg/kg/day nicotine, and were shown to be dependent on the rate of nicotine infusion with more signs being observed at the higher dose of nicotine. This model has met a number of validity criteria, including potent reversibility of abstinence signs by nicotine injection and comparative lack of signs in saline infused animals. This nicotine abstinence syndrome has also been shown to be

precipitated by injection of the nicotinic antagonists mecamylamine (s.c.; Hildebrand et al., 1999, 1997; Malin et al, 1994), dihydro-β-erythroidine (DHβE; i.c.v.; Malin et al, 1998) and hexamethonium (i.c.v.; Malin et al., 1998).

Brain stimulation reward thresholds have been shown to be a valid and reliable measure of the diminished reward and motivation associated with withdrawal from several drugs of abuse including cocaine, amphetamine, opiates and ethanol. Withdrawal from chronic nicotine resulted in elevations in brain stimulation reward thresholds (Epping-Jordan et al., 1998). These elevations in brain stimulation reward thresholds were also observed after DHβE, and mecamylamine precipitated withdrawal from nicotine (Watkins et al., 2000; Epping-Jordan et al., 1998).

A consistent symptom of nicotine withdrawal is increased anxiety and a number of groups have shown this using animal models. Costall et al. (1990b, c) showed an anxiogenic withdrawal response in the light-dark exploration test. An enhanced acoustic startle response was also seen for the first 4-5 days after nicotine withdrawal in animals receiving 0.3 and 0.6 mg/kg/day (Helton et al., 1993). Sorenson and Wilkinson (1983) demonstrated that rats given nicotine in their drinking water for 10 days showed increase startle responding 3 days following the termination of nicotine exposure. This withdrawal response has been shown to be reversed by administration of the normal daily dose of nicotine (Malin et al., 1992).

The 5-HT system is thought to be involved in the anxiogenic withdrawal response following termination of nicotine administration, as a number of drugs that modify this system have been shown to reverse this withdrawal effect. In humans, the 5-HT_{1A} receptor agonist, buspirone, has been shown to have beneficial effects on nicotine withdrawal symptoms and in helping patients achieve smoking cessation (Hilleman et al., 1994, 1992; West et al., 1991). Costall et al. (1990b, c) have shown using the light-dark exploration test that administration of the selective $5HT_3$ receptor antagonist ondansetron administered either systemically or directly into the DRN or amygdala reverses the anxiogenic withdrawal response. Rasmussen et al. (1997) have shown that during nicotine withdrawal there is an enhanced acoustic startle response that is reversed by $5HT_{1A}$ receptor antagonists such as NAN190, LY2063310 and WAY100,635.

1.12 Aims of this Thesis

Acute nicotine treatment has been shown to have both anxiolytic and anxiogenic effects that are dose-dependent (Ouagazzal et al., 1999a; File et al., 1998). Thus, the initial section of this thesis investigated the acute effects of sub-cutaneous nicotine injections on singly housed male rats at varying times after injection, using both the social interaction (Chapter 2) and the elevated plus-maze (Chapter 3) tests. These chapters also investigated whether tolerance would develop to the acute effects on anxiety after chronic treatment (7-14 days) with a low dose of nicotine (0.1 mg/kg) and whether there was a change in anxiety after withdrawal from this treatment. Social isolation has been shown to modify anxiety-related behaviours (Hall et al., 1998;

Smith et al., 1997; Fone et al., 1996; Lopes da Silva et al., 1996) and so Chapter 4 investigated whether the effects of housing conditions would modify the effects of nicotine in the two aforementioned animal tests of anxiety. Animals were either housed singly or in groups of five.

Nicotine is one of the most widely abused psychostimulant drugs in the world. Thus, Chapter 5 investigated the changes in anxiety (using the social interaction test) that occur when rats are self-administering nicotine (15 infusions of 0.03 mg/kg, totalling 0.45 mg/kg/session; i.v.) and when they are withdrawn from 4 weeks of self-administration. In order to determine if the effects seen in Chapter 5 were due to the dose of nicotine or because the animals were self-administering nicotine, Chapter 6 investigated the effects on the development of tolerance and withdrawal of chronic nicotine using different routes of administration. Rats received nicotine either by passively administered i.v. doses of nicotine in the same pattern as that used for self-administration, by once daily sub-cutaneous injections or by continuous sub-cutaneous infusion using osmotic mini-pumps. In all cases, the rats received the same daily dose of nicotine (0.45 mg/kg).

The involvement of neuroanatomical substrates in the development of tolerance to nicotine's anxiolytic and anxiogenic effects was investigated using the social interaction test. To date, the neuroanatomical site involved in mediating the anxiolytic effect of nicotine has not been elucidated. Thus, Chapter 7 investigated the role of the DRN as a site mediating the anxiolytic effect of nicotine and whether the effect was

mediated through the serotonergic system by co-administering nicotine with the 5-HT_{1A} antagonist WAY 100,635. This Chapter also explored the role of the DRN in mediating tolerance to the anxiolytic effect of nicotine. The dorsal hippocampus has been shown to be one neuroanatomical site mediating the anxiogenic effect of nicotine in the social interaction test. The anxiogenic effect is thought to be due to nicotine increasing 5-HT in this area and acting on 5-HT_{1A} receptors. Therefore, Chapter 8 investigated the involvement of the dorsal hippocampus in mediating tolerance to the anxiogenic effect of nicotine. In order to investigate a possible mechanism underlying the development of tolerance to anxiogenic effect of nicotine in the dorsal hippocampus, [^{3}H]-5-HT release was measured in superfused dorsal hippocampal slices taken from rat that had been chronically treated with nicotine.

Like nicotine, benzodiazepines have a high incidence of dependence in humans. Benzodiazepines are widely used as anxiolytics and tolerance develops to their anxiolytic effects after about 3 weeks of treatment. Thus, the final chapter investigated whether a short period of nicotine pre-treatment modified the anxiolytic effects of benzodiazepines. The dorsal hippocampus and DRN are two sites that mediate the anxiolytic effects of benzodiazepines (Nazar et al., 1999; Gonzalez et al, 1998; Gonzalez and File, 1997; Stefanski et al., 1993; Thiebot et al, 1982) and therefore midazolam was directly administered into these two brain regions. In order to determine if there were any changes in benzodiazepine receptor binding in either of these two brain areas after chronic nicotine treatment radiolabelled ligand binding studies were conducted.

CHAPTER 2

Time-course of changes in the social interaction test of anxiety following acute and chronic administration of nicotine

2.1 Introduction

Nicotinic agonists have been shown to have anxiolytic effects after acute systemic administration in many animal tests of anxiety (Brioni et al., 1994, 1993; Cao et al., 1993; Costall et al., 1989b; Vale and Green, 1986). In contrast to Brioni's report of anxiolytic effects after acute systemic administration of nicotine in the elevated plus maze (Brioni et al., 1994, 1993), Ouagazzal et al. (1999a) have reported anxiogenic effects. In the social interaction test, both anxiolytic and anxiogenic effects have been observed 30min after an acute systemic nicotine injection that are dose and test-condition dependent (File et al., 1998). Thus, a complex pattern of nicotine's effects on anxiety are seen after acute administration.

In the mouse black/white crossing test of anxiety no tolerance to the acute anxiolytic effect of nicotine (0.1mg/kg; i.p.) was found following 3, 7 and 14 days of twice daily nicotine injections (Costall et al., 1989b). However, a significant anxiogenic effect was seen 8, 48 and 96 h after withdrawal from this treatment of 14 days of twice daily injections of nicotine (Costall et al., 1989b). The anxiogenic withdrawal response is difficult to reconcile with the finding that acute nicotine treatment can also result in an

anxiogenic effect in the social interaction test. Thus, the purpose of the present study was therefore to examine whether tolerance developed to this anxiogenic effect after 4, 7 and 14 days of treatment. It also examined whether there would be changes in anxiety when the animals were tested in the social interaction test 72 h after withdrawal from a period of chronic treatment. Since the effects of nicotine 5 min after injection has not been examined, experiment 1 was a dose response study, conducted in the low light, unfamiliar (LU) test condition, to determine an anxiogenic dose of nicotine for use in subsequent experiments. From this study, the dose 0.1 mg/kg (s.c.) was selected, and experiment 2 examined the effects after 4 days of nicotine treatment. Since tolerance had not developed at this stage, experiment 3 examined whether tolerance had developed to the anxiogenic effect after 7 and 14 day's nicotine treatment and whether there were any changes when rats were tested after 72 h withdrawal from 7 and 14 day's treatment with nicotine.

As an anxiogenic effect was seen both acutely and after withdrawal, it was thought that perhaps there was a change in the effect of nicotine on anxiety with time, with an anxiogenic effect occurring shortly after injection, which over time became anxiolytic, as is the case for locomotor activity, which changes from a depressant to a stimulant effect over time (Clarke and Kumar, 1983a, b). Therefore, experiment 4 examined the time course of effects of 0.1 mg/kg (s.c.). An anxiolytic effect was observed 30 minutes after injection and the rats were therefore tested 30 min after 7 days of treatment to see whether tolerance developed to this effect.

2.2 Materials and Methods

Animals

In all experiments, male hooded Lister rats (Charles River, UK) were housed in groups of five until 5 days before testing, when all animals were singly housed. At testing, the animals weighed 150-200 g (experiment 1) and 250-300 g (experiments 2, 3 and 4). All animals were housed in the same animal room, maintained at 22°C, with lights (<50 scotopic lux) on from 0700-1900 h. Food and water were freely available.

Apparatus

The social interaction test (Figure 1.1) was a wooden box 60 x 60 cm, with 35 cm high walls; the light levels were 300 and 30 radiometric lux for the high and low light conditions, respectively. A closed circuit television camera was mounted vertically above the arena and the rats were observed on a monitor in an adjacent room by an observer who was blind to the drug treatment. The time spent in social interaction (sniffing, following and grooming the partner, boxing and wrestling) provided the measure of anxiety. The interruption of infrared beams from photocells mounted in the walls, 4.5 cm from the floor, provided an automated measure of locomotor activity (File, 1980).

Drugs

For all experiments (-)-nicotine hydrogen tartrate (Sigma) was dissolved in distilled water, in a volume of 1 ml/kg body weight. Control animals received equal volume injections of distilled water, and all injections were sub-cutaneous (s.c.).

The doses of nicotine used in experiment 1 were 0.05, 0.1 and 0.5 mg/kg (free base). It was decided from experiment 1 that the most suitable dose to use for the subsequent experiments was 0.1 mg/kg nicotine.

Procedure

Within each experiment, animals were allocated to test partners on the basis of weight, such that members of a pair did not differ by more than 10 g. On the test day pairs of rats were tested for 4.5 min and their behaviour scored by an observer with no knowledge of their drug treatment. In experiment 1, rats were tested in an unfamiliar arena, lit by low light. In all the other experiments, rats were tested in a brightly lit, familiar arena. In order to familiarise the animals with the test arena, each rat was placed singly in the brightly lit arena on the day prior to testing, for a 10 min familiarisation trial. These two test conditions were selected since they allow detection of both anxiolytic and anxiogenic effects (File et al., 1998). At the end of the test, the rats and any faecal boluses were removed and the arena wiped with a damp cloth. All animals were tested in an order randomised for drug treatment, between 0800 and 1300 h. In experiments when nicotine was given chronically, all the

animals were given daily injections of vehicle or nicotine, as appropriate, to equate handling and injection experience.

In experiments 1, 2 and 3a animals were tested 5 min after s.c. injection of nicotine or vehicle, with both animals in the pair receiving the same dose. In experiment 3b, the animals were tested uninjected and undrugged 72 h after withdrawal from 7 and 14 days nicotine treatment. In experiment 4, the animals were tested at various times after injection.

Experiment 1

Sixty-two animals were randomly allocated (n=7 or 8 pairs/group) to the following groups: vehicle, 0.05, 0.1 and 0.5 mg/kg nicotine.

Experiment 2

Thirty-two animals were randomly allocated to the following two groups (n=8 pairs/group): vehicle or 4 days nicotine (0.1 mg/kg) treatment.

Experiment 3a

Sixty-two animals were randomly allocated to the following groups (n=7 or 8 pairs/group): vehicle, acute nicotine, 7 and 14 days nicotine (0.1 mg/kg) treatment.

Experiment 3b

Forty-eight animals were randomly allocated to the following three groups (n=8 pairs/group) vehicle, 7 and 14 days of nicotine (0.1 mg/kg) treatment. The animals were first tested following an acute dose of nicotine to confirm an anxiogenic effect and were then tested uninjected and undrugged 72 h after their last s.c. injection, to examine the effects of withdrawal from chronic nicotine.

Experiment 4

Sixty-eight animals were divided into three groups to examine the effects of acute nicotine administration on the time spent in social interaction at varying times after injection. Group A (n=6 pairs/vehicle group and n=8 pairs/nicotine group) was tested 30 min after injection, group B (n=4 pairs/vehicle group and n=6 pairs/nicotine group) was tested 1 and 3 h after injection and group C (n=4 pairs/vehicle group and 6 pairs/nicotine group) 30 h after the 1st nicotine injection and then 30 min following a 2nd injection of nicotine administered on day 2. Following their 1st injection and test, both groups A and C were treated for a further 6 days with once daily injections of nicotine (0.1 mg/kg, s.c.) and then tested 30 min following their seventh injection.

Statistics

In all experiments, the scores were analysed by one-way analysis of variance (ANOVA), and comparisons between individual groups were then made with Fisher's post-hoc tests. When there were significant changes in both social interaction and

motor activity, analysis of covariance (ANCOVA) were conducted in order to determine the independence of the changes.

2.3 Results

Experiment 1

Acute nicotine administration produced a dose-dependent decrease in the time spent in social interaction [$F(3,27)=12.1$, $p<0.0001$], and post-hoc tests demonstrated that 0.1 and 0.5 mg/kg reached statistical significance ($p<0.05$ and 0.01, respectively; Table 2.1). Nicotine significantly reduced locomotor activity ($F(3,27)=12.1$, $p<0.0001$; Table 2.1), but only the 0.5 mg/kg group was significantly reduced compared with the vehicle group ($p<0.01$). Analysis of covariance confirmed that the decrease in social interaction was independent of the reductions in locomotor activity [$F(1,12)=10.6$, $p<0.01$]. However the dramatic decrease in locomotor activity was mainly secondary to the decrease in social interaction, and when the latter was accounted for, the locomotor activity was no longer significantly decreased [$F(1,12)=3.7$, $p=0.08$].

Experiment 2

After 4 days treatment with nicotine (0.1 mg/kg) there was a significant decrease in the time spent in social interaction [$F(1,14)=6.4$, $p<0.05$], but no change in locomotor activity (Table 2.2), indicating a specific anxiogenic effect.

Table 2.1 Mean (± sem) time (s) spent in social interaction and locomotor activity (beam breaks) made by rats tested 5 min after an acute dose of nicotine (0.05, 0.1 and 0.5 mg/kg s.c.) and tested in the low light, unfamiliar (LU) test condition. ** $p<0.01$ compared with vehicle control; # no longer significant after analysis of covariance (see text for details).

	Nicotine (mg/kg)			
	Vehicle	0.05	0.1	0.5
Social Interaction	87.5 ± 10.5	68.6 ± 9.0	45.9 ± 14.1 **	7.2 ± 2.7 **
Locomotor Activity	273.7 ± 22.8	286.0 ± 21.2	225.9 ± 29.7	61.0 ± 21.6 #

Table 2.2 Mean (± sem) time (s) spent in social interaction, locomotor activity (beam breaks) and number of rears after 4 days treatment with vehicle or nicotine (0.1mg/kg). Rats were tested in the high light, familiar (HF) test condition, 5 min after injection. **$p<0.01$ compared with vehicle control.

	Vehicle (s.c.) 4 Days	Nicotine (0.1 mg/kg; s.c.) 4 Days
Social Interaction	142.2 ± 9.8	103.3 ± 4.8 **
Locomotor Activity	367.6 ± 26.4	322.6 ± 27.7

Experiment 3a

In this experiment, nicotine significantly changed social interaction [$F(3,27)=4.0$, $p<0.05$] and comparisons between the individual groups showed that the acute dose (0.1 mg/kg) was significantly different from both the vehicle control ($p<0.01$) and the 7 day treatment group ($p<0.05$), see Figure 2.1. Neither the 7 nor 14 day treatment group differed from the control group. In this experiment, the animals treated with acute nicotine also showed a decrease in locomotor activity, but after analysis of covariance this was no longer significant [Mean ± sem: vehicle = 362.6 ± 23.6; acute nicotine, 264.2 ± 56.7; $F(1,12)=1.6$, $p>0.2$].

Experiment 3b

Withdrawal from 7 and 14 day's treatment with nicotine (0.1 mg/kg/day) significantly decreased the time spent in social interaction [$F(2,21)=5.2$, $p<0.05$], and both groups showed significant decreases in the time spent in social interaction ($p<0.05$ and $p<0.01$, respectively; Figure 2.2) compared with controls. There was no change in locomotor activity in these groups (Table 2.3).

Table 2.3 Mean (± sem) locomotor activity (beam breaks) made by rats injected with nicotine (0.1 mg/kg s.c.) after 72 h withdrawal from 7 (7W) and 14 (14W) days treatment with nicotine (0.1 mg/kg s.c.). Rats were tested in the high light, familiar (HF) test condition, 5 min after injection.

	Vehicle	7W	14W
Locomotor Activity	367.6 ± 26.4	411.6 ± 24.3	364.0 ± 21.7

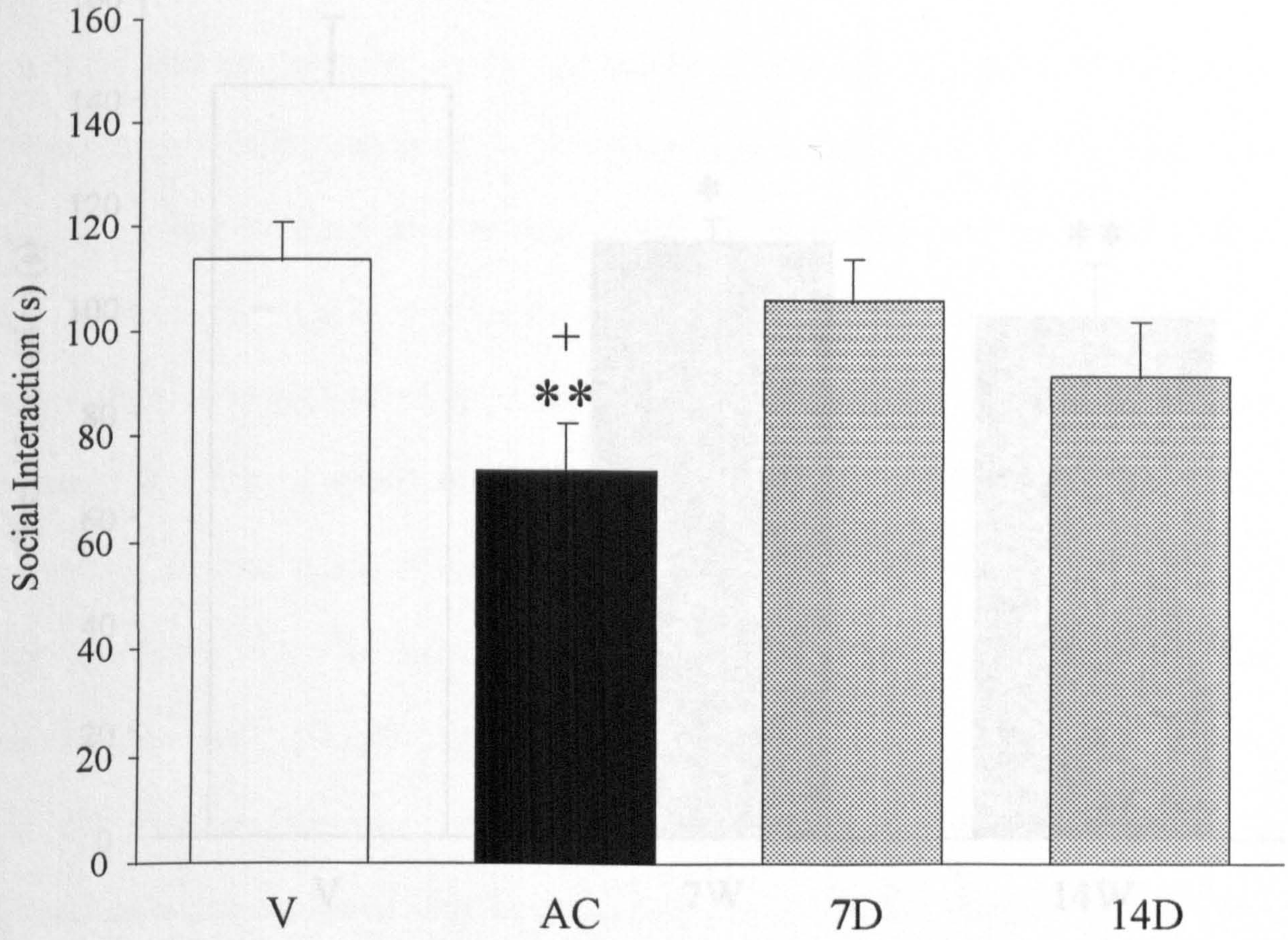

Figure 2.1 Mean (± sem) time spent in social interaction by rats injected with nicotine (0.1 mg/kg s.c.) after vehicle, acute nicotine (AC), or 7 (7D) and 14 (14D) days nicotine. All doses of nicotine were 0.1 mg/kg s.c. and rats were tested 5 min after injection. ** $p<0.01$ compared with vehicle control, $^{+}$ $p<0.05$ compared with 7D group.

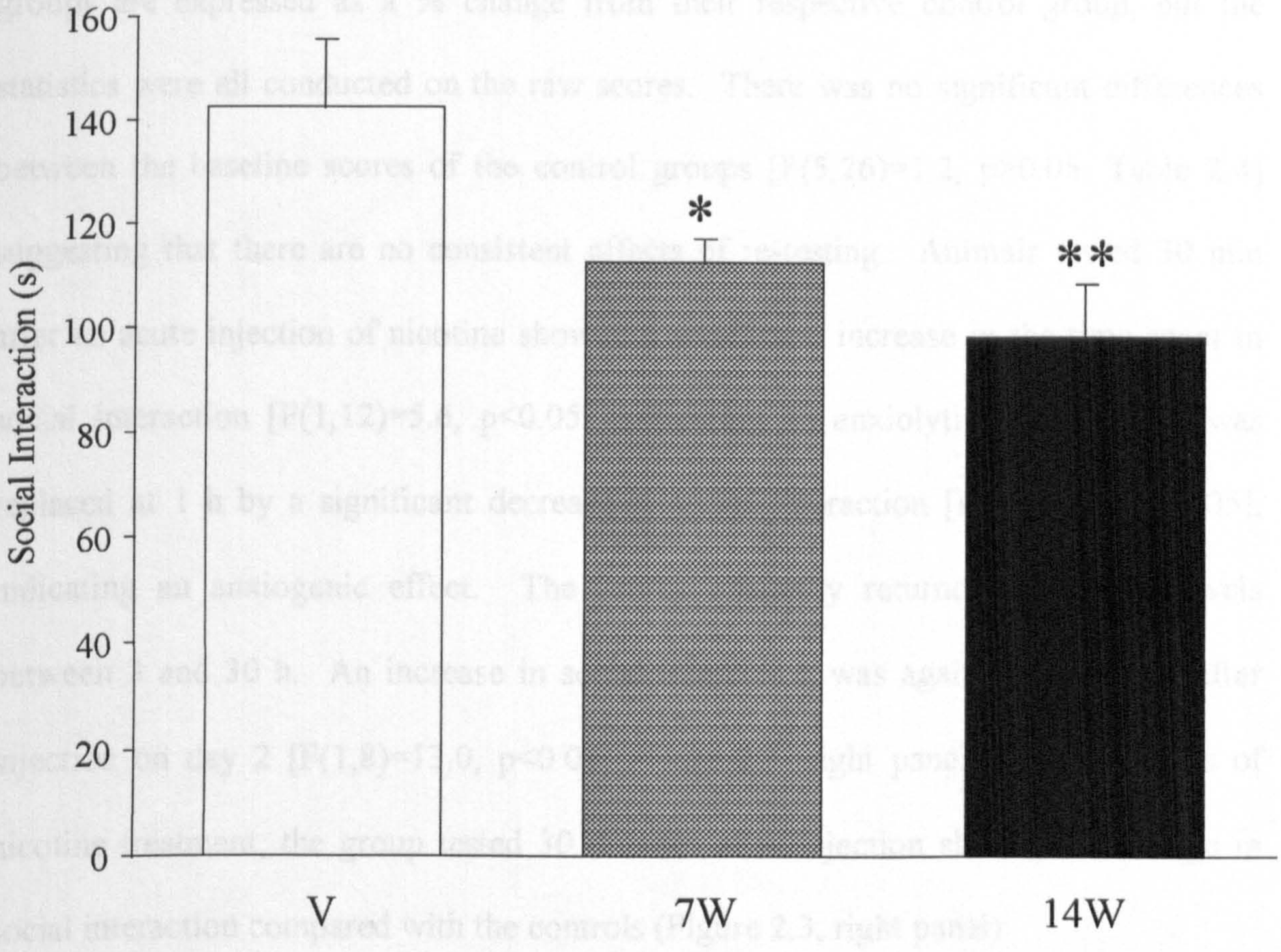

Figure 2.2 Mean (± sem) time spent in social interaction by rats injected with nicotine (0.1 mg/kg s.c.) after 72 h withdrawal from 7 (7W) and 14 (14W) days treatment with nicotine. Rats were tested in the high light familiar (HF) test condition, 5 min after injection. * $p<0.05$ and ** $p<0.01$ compared with vehicle control.

Experiment 4

The left panel in Figure 2.3 shows the time-course of effects on social interaction following an acute injection of nicotine. For illustrative purposes the scores of all the groups are expressed as a % change from their respective control group, but the statistics were all conducted on the raw scores. There was no significant differences between the baseline scores of the control groups [$F(5,26)=1.2$, $p>0.05$; Table 2.4] suggesting that there are no consistent effects of re-testing. Animals tested 30 min after an acute injection of nicotine showed a significant increase in the time spent in social interaction [$F(1,12)=5.6$, $p<0.05$], indicating an anxiolytic effect. This was replaced at 1 h by a significant decrease in social interaction [$F(1,8)=5.3$, $p=0.05$], indicating an anxiogenic effect. The scores gradually returned to control levels between 3 and 30 h. An increase in social interaction was again seen 30 min after injection on day 2 [$F(1,8)=13.0$, $p<0.01$; Figure 2.3, right panel]. After 7 days of nicotine treatment, the group tested 30 minutes after injection showed no change in social interaction compared with the controls (Figure 2.3, right panel).

Table 2.4 Mean (± sem) time (s) spent in social interaction by rats treated with vehicle (in experimental groups A, B and C) and tested 30 min, 1, 3, or 30h after a single injection, or 30 min after their 2nd or 7th injection. All animals were tested in the high light familiar (HF) test condition.

	1st Injection				2nd Injection	7th Injection
Time after Injection	30 min	1 h	3h	30 h	30 min	30 min
Group	A	B	B	C	C	A+C
Social Interaction (s)	178.0 ± 8.5	207.4 ± 18.7	174.0 ± 15.7	187.0 ± 9.0	221.7 ± 15.0	182.1 ± 15.5

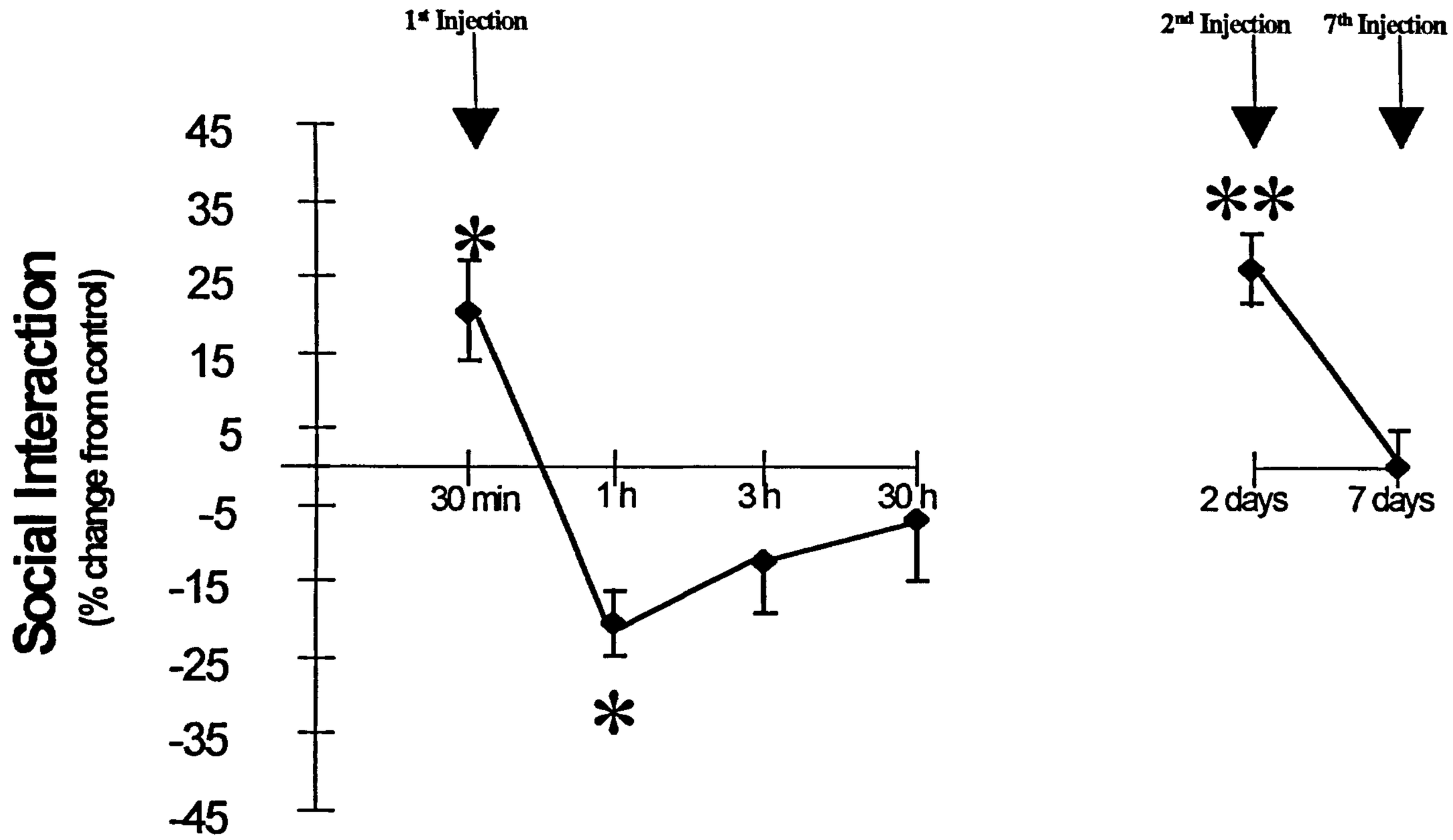

Figure 2.3 Mean (± sem) % change in time spent in social interaction compared to the respective vehicle controls of rats following either an acute nicotine injection (Panel A) or in animals following either a 2nd or 7th injection (Panel B) following various times after injection. Animals in Group A were tested 30min after their 1st and 7th injection; Group B were tested 1 and 3hr after injection, and Group C were tested 3hr after their injection, and 30min following their 2nd and 7th. The data presented following the 7th injection has been combined for the two groups tested following the 7th injection. * $p<0.05$ and ** $p<0.01$ compared with vehicle control.

The animals that were tested 30 min after their first dose of nicotine showed a significant decrease in locomotor activity [vehicle = 315.5 ± 18.7 and acute nicotine = 254.9 ± 19.5; $F(1,12)=4.8$, $p<0.05$]. In contrast, the animals that were tested 30 min after their second nicotine injection showed an increase in locomotor activity. However, after analysis of covariance the increase in locomotor activity was no longer significant [$F(1,7) = 4.0$, $p<0.1$], suggesting that it was, at least in part, secondary to the increase in social interaction. None of the other groups showed any change in locomotor activity, compared with their respective control group.

2.4 Discussion

This study has shown that after an acute administration of a low dose of nicotine (0.1 mg/kg; s.c.) both anxiolytic and anxiogenic effects can be observed in the social interaction test, at different times after injection. Although the animals tested after 5 min were investigated in a separate experiment from the other time points, the acute anxiogenic effect was seen in both the LU and HF test condition (Experiments 1 and 3a). The effects that were seen 5 min, 30 min and 1 h after a nicotine injection were observed in animals that had been tested only once. File et al. (1998) have shown that 30 min after an i.p. injection of nicotine, high doses can have anxiogenic, and low doses anxiolytic, effects in this test. This suggests that the effect of an acute dose of nicotine is both dose- and time-dependent. Nicotine has also been shown to have complex effects on locomotor activity and intracranial self-stimulation, with both decreases and increases in these behaviours observed (Herberg et al., 1993; Clarke and Kumar, 1983a, b), dependent on both the dose and time after administration.

Interestingly, it appears that the time-course of change from an anxiogenic to an anxiolytic effect is similar to that seen in the change from decreases to increases in locomotor activity and intracranial self-stimulation.

The decreased social interaction following acute nicotine treatment and during withdrawal from chronic treatment was not accompanied by decreases in locomotor activity. That nicotine withdrawal does not produce changes in locomotor activity is in agreement with previously published results (Helton et al, 1993; Clarke and Kumar, 1983a). The increased social interaction following the first nicotine injection was accompanied by a decrease in locomotor activity, providing a very clear dissociation between the two measures. Only after the second nicotine injection were there increases in both measures, but analysis of covariance showed that the increase in locomotor activity was secondary to the increased social interaction. In the black-white crossing test of anxiety, no tolerance was found to the anxiolytic effect of nicotine (Costall et al, 1989b), but this could be because measures of anxiety in this behavioural test are heavily contaminated by changes in locomotor activity. Tolerance does not develop to the locomotor stimulant effect of nicotine, and this stimulatory effect becomes more marked with chronic treatment (Shoaib et al., 1997; Ksir et al., 1987; Schwartz and Kellar, 1985).

After 7 days of chronic nicotine treatment, tolerance was found to both the anxiogenic and anxiolytic effects that were observed in the social interaction test at 5 and 30 min after acute administration, respectively. It is thought that an oppositional process may

account for tolerance to the anxiolytic effect since after 7 and 14 days of treatment as there was an anxiogenic withdrawal effect at 72 h. Further evidence for the involvement of such a mechanism is that there appears to be a rebound anxiogenic effect 1 h after injection suggesting that after an acute injection an oppositional process comes into play. An oppositional mechanism involves the recruitment of processes that oppose the anxiolytic effect of the acute administration of nicotine, leading to behavioural tolerance (Young and Goudie, 1995). Following drug withdrawal, these processes work unopposed, resulting in the appearance of behavioural changes, such as the decrease in social interaction. Such a mechanism also seems to underlie the development of tolerance to the anxiolytic effects of benzodiazepines (e.g. File et al., 1987a). In contrast to an oppositional mechanism which seems to underlie the development of tolerance to the anxiolytic effects of nicotine, the mechanism underlying tolerance to the anxiogenic effects is more likely to be a decremental one. A decremental process of tolerance is one in which the impact of a drug is reduced (e.g. by receptor desensitisation), but which is without behavioural consequence in the absence of the drug (Young and Goudie, 1995).

If the anxiogenic effect observed 5 min after injection were subject to rapid desensitisation (Fenster et al., 1997), this would then reveal the anxiolytic effect, as long as this was not also subject to a similar desensitisation. It is certainly possible that different behavioural effects of nicotine will be subject to different rates of desensitisation since there is evidence that different nicotinic subunit assemblies show different rates of desensitisation and recovery (Fenster et al., 1997). If a decremental

process underlies the development of tolerance to the anxiogenic effect of nicotine, this could result from a deactivation of receptors. Chronic exposure to nicotine irreversibly inactivates some nicotinic subunits, whereas others are much less affected (Olale et al., 1997). Thus, a deactivation of subunits in a particular brain region, such as the dorsal hippocampus or lateral septum, could lead to tolerance to the anxiogenic effect. If other subunits in a different brain region were mediating the anxiolytic effect, these might be subject to less deactivation after chronic treatment, with an oppositional mechanism mediating the tolerance to this action and the anxiogenic withdrawal response.

In summary, the results of the present experiments have revealed a complex pattern of changes in social interaction induced by nicotine, with both anxiolytic and anxiogenic effects emerging at different time-points. After 7 days of nicotine treatment, tolerance was found to both the initial anxiogenic effect and anxiolytic effect. Further experiments will be needed to determine which brain regions are involved in mediating tolerance to these effects.

CHAPTER 3

Tolerance to nicotine's effects in the elevated plus-maze and increased anxiety during withdrawal

3.1 Introduction

The effects of nicotine on anxiety are unusual in that it can have both anxiolytic and anxiogenic effects in animal tests (Ouagazzal et al., 1999a; File et al., 1998; Brioni et al., 1993; Cao et al., 1993; Costall et al., 1989b; Vale and Green, 1986), in non-smoking volunteers (File et al., 2000a; Newhouse et al., 1990) and in smokers (Netter et al., 1998; Ikard et al., 1969). In the social interaction test, the direction of nicotine's effects have been shown to be dependent on dose, with low doses being anxiolytic and high doses anxiogenic. The anxiogenic effect of nicotine has been shown to be mediated by the dorsal hippocampus and lateral septum (Cheeta et al., 2000a; Kenny et al., 2000b; Ouagazzal et al., 1999b). In the rat elevated plus-maze test of anxiety, both anxiolytic (Brioni et al., 1994) and anxiogenic (Ouagazzal et al., 1999a) effects have been reported, but in this test the direction of nicotine's effects was not dose-related. Chapter 2 showed that the direction of nicotine's effects on anxiety, as measured in the social interaction test depends on the time since injection. Nicotine (0.1 mg/kg; s.c.) had an anxiogenic effect 5 min after injection, but an anxiolytic action after 30 min (Chapter 2).

The effects of nicotine (0.1 mg/kg) on anxiety have not yet been examined in the plus-

maze 5 min after injection. The purpose of the present experiment was therefore to examine the effects of nicotine (0.1 mg/kg; s.c.) 5 and 30 min after injection of a single dose and after a period of chronic treatment. In the social interaction test, after a week of pretreatment, tolerance developed to both the anxiogenic effect observed 5 min after injection and to the anxiolytic effect found at 30 min (Chapter 2), but to date tolerance to the effect of nicotine has not been examined in the plus-maze. In order to determine whether tolerance was due to an oppositional mechanism (Young and Goudie, 1995), animals were also tested undrugged 24h after the last of the chronic injections. Increased anxiety has been reported on withdrawal from nicotine in animal tests (Chapter 2; Costall et al, 1989b), in smokers (Parrott and Garnham, 1998; Parrot et al., 1996; West and Russell, 1985; Shiffman and Jarvik, 1976) and in those withdrawing from nicotine gum (Hughes et al., 1990; Keenan et al., 1989). The dorsal hippocampus does not seem to play a role in the acute effects of nicotine in the plus-maze (Ouagazzal et al., 1999b), but it does seem to play a very general role in mediating stress-induced changes in a variety of other test situations (File et al., 2000b). Thus, this chapter also examined whether a low dose of nicotine would be effective when administered to the dorsal hippocampus of rats in 24h withdrawal from nicotine.

3.2 Materials and Methods

Animals

Male hooded Lister rats (Charles River, Margate, Kent, UK) weighing between 220-250g were housed singly. The animals in the withdrawal study that had undergone

surgery were allowed to recover for 4 days prior to the start of chronic injections. Food and water were freely available, and the room in which they were housed was lit with dim light and maintained at 22°C. Lights were on from 0700-1900 h.

Elevated Plus-Maze Test

The elevated plus maze (Figure 1.2) was made of wood and consisted of two opposite open arms 50x10cm, and two opposite equal sized arms enclosed by 40cm high walls. The arms were connected by a central 10x10cm square, and thus the maze formed a "plus" shape. The maze was elevated 50cm from the floor and lit by dim light. A closed-circuit TV camera was mounted vertically over the maze, and the behaviour was scored from a monitor in an adjacent room by an observer who was blind to the drug treatment. The number of entries onto, and the times spent on, open and closed arms were recorded by an observer blind to the drug treatment. Four paws into, and two paws out of, an arm defining an arm entry and exit, respectively. The percentage number of open arm entries [open entries/(open+closed entries)x100] was calculated, as was the percentage of time spent on the open arms. The percentage of entries onto, and the percentage of time spent on the open arms of the maze provide the measure of anxiety, and the number of closed arm entries provides the best measure of locomotor activity in this test (File, 1992; Pellow et al., 1985). At the end of each trial, any faecal boluses were removed from the maze, which was wiped clean with a damp cloth.

Surgery

Rats were anaesthetised by inhalation of 3% isofluorane (May and Baker, Dagenham, Essex, UK) in oxygen and positioned in the stereotaxic frame (Kopf Instruments, Tujunga, California, USA). The skull was exposed and the incisor bar adjusted such that bregma and lambda were at the same height. Three indentations were made in the skull to accommodate screws, which, together with the application of dental cement, held the cannulae in place. For bilateral cannulation of the dorsal hippocampus, 7 mm long steel guide cannulae (23 gauge, Cooper's Needle Works Ltd, Birmingham, UK) were positioned at 3.3 mm posterior to bregma, ± 2.4 mm lateral, and -1.2 mm vertical, thus siting them 2 mm above the target area (according to the atlas of Paxinos & Watson, 1986). Cannulae were kept patent using 7 mm long stainless steel stylets (30 gauge, Cooper's Needle Works Ltd, Birmingham, UK). On the test day, rats were gently wrapped in a cloth and injected using needles constructed from 30 gauge steel tubing that extended 2mm below the tip of the in-dwelling cannulae, into the dorsal hippocampus. In order to accustom the animals to handling and to keep the stylets patent, each day following surgery the rats were gently wrapped in a cloth and the stylets were replaced.

Drugs and chemicals

For the chronic subcutaneous injections, (-)-nicotine hydrogen tartrate (Sigma, Poole, UK) was dissolved in distilled water, in a volume of 1 ml/kg body weight and a dose of

0.1 mg/kg was used; control animals received equal volume injections of distilled water.

For the central injections, (-)-nicotine hydrogen tartrate was dissolved in artificial cerebrospinal fluid (aCSF) of the following composition (in mM): 126.6 NaCl, 27.4 $NaHCO_3$, 2.4 KCl, 0.5 KH_2PO_4, 0.89 $CaCl_2$, 0.8 $MgCl_2$, 0.48 Na_2HPO_4 and 7.1 glucose, pH 7.4. Injections were 0.5µl, and were made over a period of 30 sec using a CMA/102 microdialysis pump (Biotech Instruments Ltd, Stockholm, Sweden) and the needles were left in position a further 30 sec to allow drug diffusion; control animals received 0.5µl infusions of aCSF. All doses are given as free base.

Behavioural Testing

Development of tolerance

Forty-eight animals were randomly allocated to the following drug groups: vehicle, acute nicotine (0.1 mg/kg) and 7 days of nicotine (0.1 mg/kg/day) and in each group half were tested 5 min and half 30 min after injection. Because an anxiolytic effect emerged in the animals that had been treated for 7 days with nicotine and were tested after 5 min, a second group of animals was randomly allocated to the following groups: vehicle (n=9) and 14 days of nicotine (n=8; 0.1 mg/kg/day) and tested 5 min after the last injection to see if tolerance occurred to the anxiolytic effect after the longer pretreatment period.

Reversal of nicotine withdrawal response in the elevated plus-maze

Animals were randomly allocated to pretreatment with either vehicle or nicotine (0.1 mg/kg/day, s.c.) for 6 days. On the 7th day, no s.c. injections were given but rats from both pretreatment groups were randomly assigned to be tested 3 min after a bilateral injection into the hippocampus with aCSF or (-)-nicotine (5ng). The numbers in each group ranged from 7-9 after verification of the cannula placements.

Histology

At the end of behavioural testing the cannulated animals were sacrificed, the brains removed and the injection sites verified histologically (Paxinos and Watson, 1986) by a person blind to drug treatment. Figure 3.1, depicting coronal slices through the dorsal hippocampus, shows the site of the injections for the rats whose data were included in the statistical analysis.

Statistics

The data were analysed with one-way ANOVA and comparisons with individual groups were then made with Fisher's post-hoc tests; it is the significances of these that are shown in the figure and table. Because of the large number of zero scores in the withdrawal group, this group was compared with other groups using Mann-Whitney U-tests (although for ease of comparison all the scores in Figure 3.3 are presented as means ± sem).

Figure 3.1 Diagrammatic representation of coronal sections (3.14 to 3.6 mm posterior to bregma) through the rat brain showing the placements accepted as falling within the dorsal hippocampus (filled circles).

3.3 Results

Development of tolerance

In animals that were tested 5 min after s.c. injection there was a significant effect of nicotine (0.1 mg/kg) on the percentage of time spent on the open arm [$F(2,20)=8.9$, $p<0.01$] and the percentage of open arm entries [$F(2,20)=3.5$, $p=0.05$]. This arose because, although acute administration was without effect, the rats tested after their 7th injection with nicotine showed a significant increase in both measures ($p<0.01$ and $p<0.05$, respectively), compared with both the vehicle control group and the acute nicotine group, see Figure 3.2. There were no significant effects of nicotine on the number of closed arm entries [$F(2,20)=1.0$], see Figure 3.2. Thus, a specific anxiolytic effect had emerged after 7 days of chronic treatment when rats were tested 5 min after nicotine (0.1 mg/kg s.c.) injection. However, after 14 days of pretreatment, tolerance developed to this anxiolytic effect and nicotine was without effect on the percentage of time spent on the open arms [$F(1,15)=0.3$] or the percentage of open arm entries [$F(1,15)<0.1$], see Figure 3.2. However, it can be seen from Figure 3.2 that the animals that had received 14 days of nicotine injections showed a significant increase in the number of closed arm entries [$F(1,15)=9.9$, $p<0.01$] compared with vehicle controls.

In animals that were tested 30 min after injection there was a significant effect of nicotine on the percentage of time spent on the open arms [$F(2,22)=3.6$, $p<0.05$] and the percentage of open arm entries [$F(2,22)=5.2$, $p<0.05$], but in this case the significance arose because of the significant reductions in these measures caused by the acute administration of nicotine ($p<0.05$ and $p<0.05$, respectively), compared with both

the control group of animals and the chronic nicotine group, see Figure 3.2. Thus, after 7 injections, tolerance had developed to the anxiogenic effect of nicotine. The acute administration of nicotine did not change the number of closed arm entries, but there was a significant effect on the number of closed arm entries [$F(2,22)=3.7$, $p<0.05$], due to the rats tested after their 7th injection having a decrease compared with vehicle controls ($p<0.05$, Figure 3.2).

Reversal of nicotine withdrawal response

When rats were withdrawn for 24h after 6 days of nicotine pretreatment, there was a significant anxiogenic effect, shown by a decrease in the percentage of time spent on the open arms ($U=3$, $p<0.01$) and the percentage of open arm entries ($U=10$, $p<0.05$). There was no change in the number of closed arm entries [$F(1,15)=3.2$]. Bilateral administration of nicotine (5ng) into the dorsal hippocampus significantly reversed the withdrawal response on both measures ($U=9$, $p<0.05$ for % time and $U=11$, $p<0.01$ for % entries), see Figure 3.3. In the vehicle-pretreated animals this dose of nicotine administered to the dorsal hippocampus was without effect on the percentage of time spent on the open arms [$F(1,15)=0.3$], the percentage of open arm entries [$F(1,15)=0.3$] or the number of closed arm entries [$F(1,15)=0.7$], see Figure 3.3.

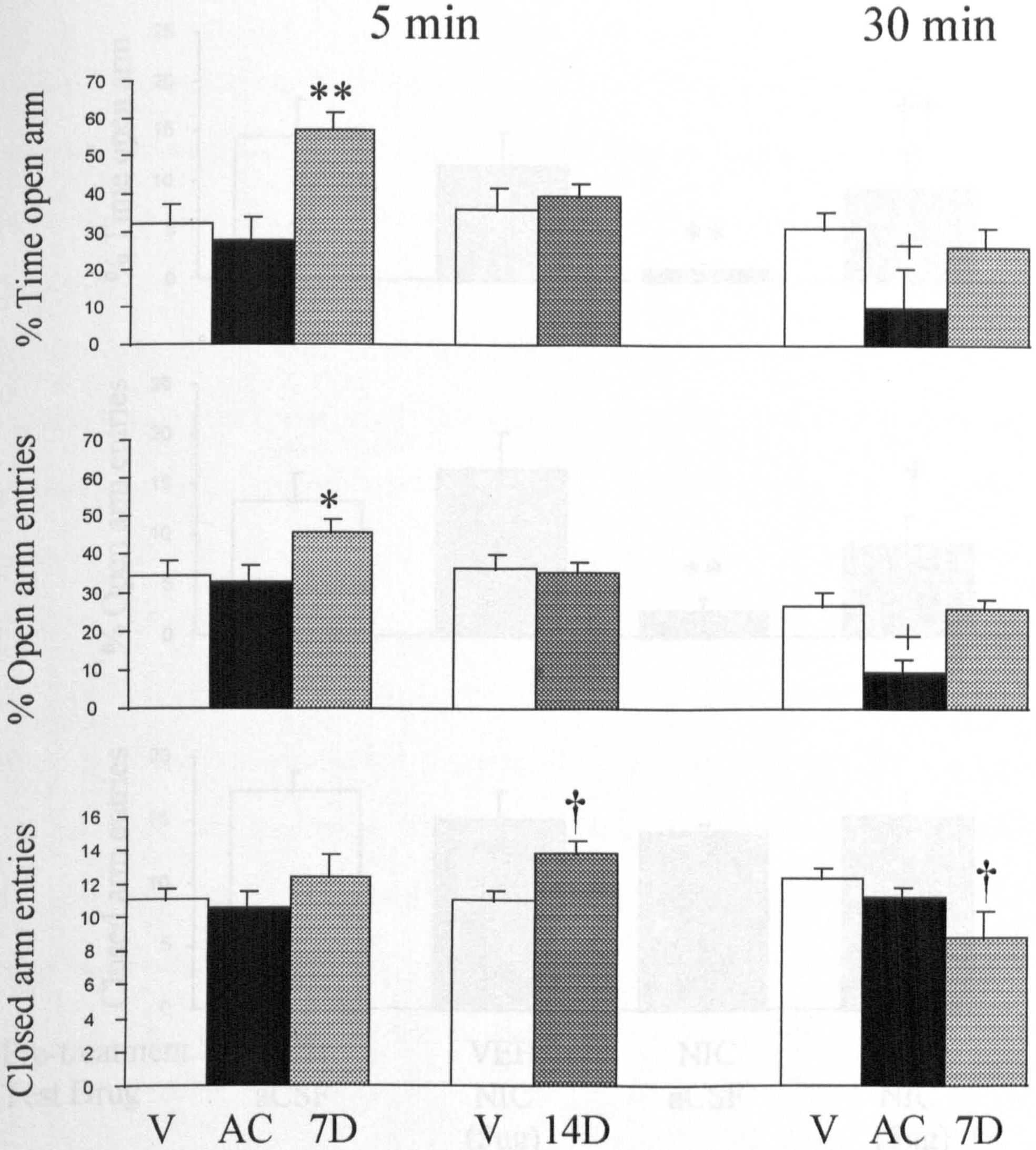

Figure 3.2 Mean (± sem) percentage time spent on open arms, percentage open arm entries and number of closed arm entries in the plus-maze 5 and 30 min after s.c. injection of vehicle (V), acute nicotine (0.1 mg/kg; AC), or 7 days of nicotine (0.1 mg/kg; 7D) and 5 min after s.c. injection of vehicle or 14 days of nicotine (0.1 mg/kg; 14D). * $p<0.05$ and ** $p<0.01$ compared with the vehicle control and acute nicotine group, $^{+}$ $p<0.05$ compared with the vehicle control and chronic nicotine group, †$p<0.05$ compared with the vehicle control .

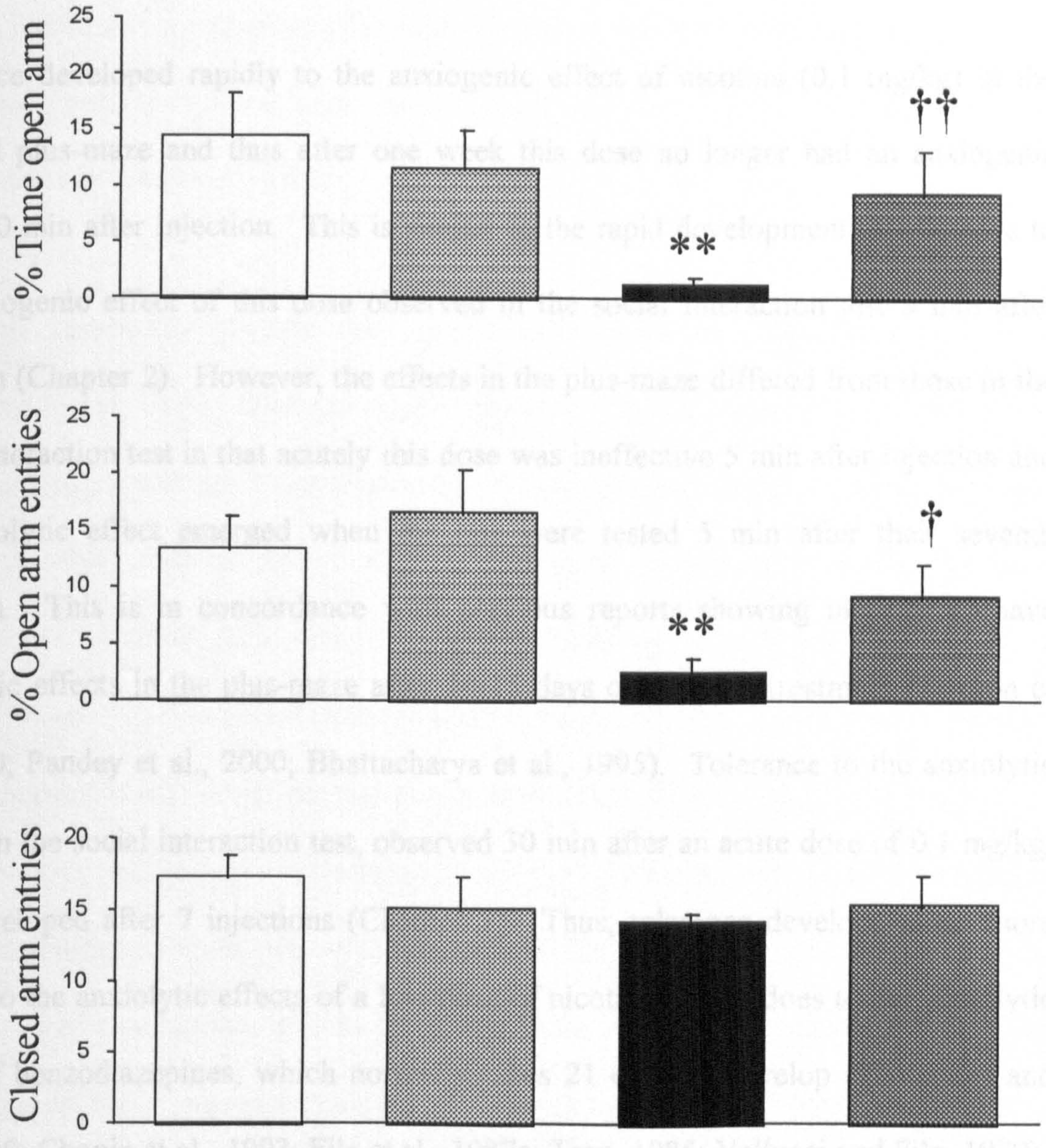

Figure 3.3 Mean (±sem) percentage of time spent on open arms, percentage of open arm entries and number of closed arm entries in the plus-maze in animals pretreated for 6 days with vehicle or nicotine (0.1 mg/kg/day; s.c.) and tested 24h later, 3 min after bilateral dorsal hippocampal injections of vehicle (aCSF) or nicotine (5ng). *$p<0.05$ and ** $p<0.01$ compared with the vehicle control (VEH, aCSF), †$p<0.05$ and ††$p<0.01$ compared with the withdrawal group (NIC, aCSF).

3.4 Discussion

Tolerance developed rapidly to the anxiogenic effect of nicotine (0.1 mg/kg) in the elevated plus-maze and thus after one week this dose no longer had an anxiogenic effect 30 min after injection. This is similar to the rapid development of tolerance to the anxiogenic effect of this dose observed in the social interaction test 5 min after injection (Chapter 2). However, the effects in the plus-maze differed from those in the social interaction test in that acutely this dose was ineffective 5 min after injection and an anxiolytic effect emerged when the rats were tested 5 min after their seventh injection. This is in concordance with previous reports showing nicotine to have anxiolytic effects in the plus-maze after 14-15 days of nicotine treatment (Ericson et al., 2000; Pandey et al., 2000; Bhattacharya et al., 1995). Tolerance to the anxiolytic effects in the social interaction test, observed 30 min after an acute dose of 0.1 mg/kg, also developed after 7 injections (Chapter 2). Thus, tolerance develops much more rapidly to the anxiolytic effects of a low dose of nicotine than it does to the anxiolytic effect of benzodiazepines, which normally takes 21 days to develop (Fernandes and File, 1999; Chopin et al., 1993; File et al., 1987a; Treit, 1985; Vellucci and File, 1979).

There was a dissociation in the time-course of changes in locomotor activity, as measured by closed arm entries, and the measures of anxiety. Thus, in the rats tested 5 min after injection, there were no changes in locomotor activity when the anxiolytic effect emerged at 7 days, but after 14 days when there were no changes in the measures of anxiety there was evidence of locomotor stimulation. This increase in locomotor activity after chronic nicotine treatment is in accordance with other studies (Ericson et

al., 2000; Clarke and Kumar, 1983a, b). In the rats tested 30 min after injection, there were no changes in locomotion after acute treatment, when nicotine had an anxiogenic effect, but a reduction in locomotor activity occurred after 7 days, when there was no change in the measures of anxiety.

When rats were tested 24h after the last of 6 daily injections a significant anxiogenic effect was noted, as has been previously reported (Bhattacharya et al., 1995). This anxiogenic effect was not accompanied by any change in locomotor activity which is consistent with other studies that have measured locomotor activity 24h after withdrawal of nicotine (Robinson et al., 1994; Helton et al., 1993). The studies in which a decrease in locomotor activity was found at 24h after withdrawal of nicotine used higher doses and longer periods of treatment (Hildebrand et al., 1999; Fung et al., 1996). As was reported by Bhattacharya et al. (1995), we observed no somatic signs of withdrawal in our animals, but the fact that the anxiogenic effect could be reversed by an injection of nicotine strengthens the interpretation that it is a withdrawal response. An anxiogenic effect has not found as of yet at this time-point in the social interaction test, but does occur at 72h (Chapter 2). In the mouse black-white crossing test an anxiogenic response was observed 8-96h after withdrawal from 14 days of twice daily nicotine (0.1 mg/kg/day; Costall et al., 1989b). Thus, the duration and timing of these withdrawal responses may depend both on the particular test and on the duration of treatment. However, it is clear that a withdrawal response can be observed following a relatively short period of treatment with a low dose of nicotine. Again, this contrasts with the effects of the benzodiazepines, where increased anxiety is usually only

observed after withdrawal from 3 weeks of treatment (Ward and Stephens, 1998; Andrews et al., 1997; Chopin et al., 1993; File et al., 1991a; File and Andrews, 1991; File et al., 1987a).

The incidence of a withdrawal response in the opposite direction to the acute effects of a drug is an indication of an oppositional mechanism of tolerance. Thus, benzodiazepines initially have an anxiolytic effect, tolerance develops to this and an anxiogenic response is seen on drug withdrawal. A similar pattern can be seen following repeated injections with the anxiogenic drug, pentylenetetrazole, where an anxiolytic effect is seen on withdrawal (File et al., 1996b). This is not the pattern seen in the elevated plus-maze or the black-white crossing test (Costall et al., 1989b) where both the acute response to nicotine and that seen during withdrawal are in the same direction, i.e. increased anxiety. Furthermore, a withdrawal response was observed after 6 days of treatment, at the same time that an anxiolytic effect could be seen in response to a nicotine injection. In the social interaction test, tolerance was observed after 6 days to the anxiogenic effect, but in this test no withdrawal response was found at the 24h time-point. It therefore seems unlikely that an oppositional mechanism underlies the development of tolerance to the anxiogenic effects of nicotine in any of the animal tests thus far investigated.

Whilst a decremental mechanism of tolerance can account for the development of tolerance, without the occurrence of a withdrawal response, it alone cannot explain the incidence of a withdrawal response in the same direction as the acute effect. One

possibility is that the withdrawal response is mediated by changes in a neural system different from that which is manifesting the changes of tolerance. This possibility is strengthened by our finding that although dorsal hippocampal administration of nicotine was without effect in the control animals, it was able to reverse the effects of nicotine withdrawal. In the present experiment only a single dose of nicotine was investigated, but other studies have shown a wide range of doses to be ineffective in the plus-maze (Cheeta et al., 2000a; Ouagazzal et al., 1999a). One area that mediates the anxiogenic effects of acute nicotine is the lateral septum (Cheeta et al., 2000a, b) and there seems to be a reciprocal inhibition between the dorsal hippocampus and the lateral septum in mediating behaviour in the plus-maze. The 5-HT_{1A} receptors have been implicated in this anxiogenic effect, because the co-administration into the lateral septum with the 5-HT_{1A} antagonist WAY 100,635, reversed the anxiogenic effect of nicotine (Cheeta et al., 2000b). Thus, when the baseline scores are low (e.g. 10% open arm entries) a 5-HT_{1A} receptor agonist administered to the dorsal hippocampus has an anxiolytic effect (Menard and Treit, 1998), whereas with higher baseline scores (30%) neither benzodiazepines nor a 5-HT_{1A} receptor agonist has any action (Gonzalez et al., 1998; File et al., 1996b). In contrast, when baseline scores are high an anxiogenic effect can be seen after administration of a 5-HT_{1A} receptor agonist to the lateral septum (Cheeta et al., 2000a), whereas it is without effect if baseline scores are low (Menard and Treit, 1998). The dorsal hippocampus has been implicated in the anxiogenic effect that can be detected in the plus-maze after restraint stress (Netto and Guimaraes, 1996; McBlane and Handley, 1994; Titze-de-Almeida et al., 1994) and in the stress-induced decrease in locomotor activity (Carli et al., 1993). It is therefore

possible that the anxiogenic response observed during nicotine withdrawal is an example of a wide range of stress-induced responses in which the dorsal hippocampus plays a role. Thus, the nicotine-cholinergic system in this brain region may be implicated in several stressful situations and withdrawal from nicotine may have been stressful because it constituted a major change in state. It would be interesting to see whether dorsal hippocampal administration of nicotine could reverse the anxiogenic effect seen after restraint stress in the elevated plus-maze.

The results of this study show that chronic administration of a low dose of nicotine results in the rapid development of tolerance to its acute anxiogenic effects and an anxiogenic response on drug withdrawal that could be reversed with an intra-hippocampal injection of nicotine.

CHAPTER 4

Social Isolation Modifies Nicotine's Effects In Animal Tests Of Anxiety

4.1 Introduction

Calming and anxiety-reducing effects are frequently cited by smokers as reasons for their smoking, and this is particularly prevalent amongst teenage girls (Royal College of Physicians, 2000). However, some have queried whether nicotine really does have anxiolytic effects, other than reversing the increased anxiety that results from nicotine withdrawal (Heishman et al, 1994; West, 1993). Indeed, there is evidence that under some circumstances nicotine can actually increase stress and anxiety (Netter et al, 1998; Parrott et al, 1996; Newhouse et al, 1990). The data from the previous two chapters show that even following acute administration, nicotine has complex effects on anxiety.

Anxiolytic effects of nicotine have been reported in several experimental models of anxiety in both mice (Brioni et al, 1993; Cao et al, 1993; Costall et al, 1989b), and rats (Brioni et al, 1994; Vale & Green, 1986). In the social interaction test of anxiety, the effects of nicotine in rats have been found to be dose- and time-dependent (Chapter 2; File et al, 1998). Both anxiolytic and anxiogenic effects have also been found in rats tested in the elevated plus-maze. However, it is less clear in this test that the effects are dependent on dose. Ouagazzal et al (1999a) found low doses (0.001-0.1mg/kg i.p)

were ineffective, but high doses had an anxiogenic effect (0.5-1mg/kg i.p.). In contrast, Brioni et al (1994) reported an anxiolytic action with 0.3 mg/kg nicotine. It is possible that this discrepancy can be explained by intrinsic differences between the rat strains that were used in these two studies. It is also possible that differences in the housing conditions can explain some of the discrepancies in the literature, especially between the findings in rats and mice. Anxiolytic effects have been universally reported in mice, where group housing has always been used (Brioni et al, 1993; Cao et al, 1993; Costall et al, 1989b), whereas anxiogenic effects have tended to dominate in rat studies which have used single housing (Chapter 3; Ouagazzal et al., 1999a).

Social isolation has been shown to modify anxiety-related behaviours. In the elevated plus-maze, an anxiogenic profile and a greater sensitivity to the anxiolytic effects of diazepam was seen in rats that were reared in isolation from weaning (Fone et al, 1996; Lopes da Silva et al, 1996; Wright et al, 1991; Morinan & Parker, 1985). Isolation-reared rats have also been found to be more sensitive to the effects of amphetamine, cocaine and ethanol (Hall et al, 1998; Smith et al, 1997; Fowler et al, 1993; Jones et al., 1990). The effects of a short period of isolation housing in adult rats have not been extensively investigated. Singly housed rats showed greater locomotor stimulation in response to amphetamine (Ahmed et al., 1995) and enhanced chloride uptake in response to GABA and flunitrazepam (Thielen et al, 1993). In contrast, pair-housed adult rats were more sensitive than singly housed rats to the anxiolytic effects of diazepam in the social interaction test of anxiety (Gardner & Guy, 1984). The purpose of the present experiments was therefore to determine whether the housing conditions

of adult rats would modify the effects of nicotine in two animal tests of anxiety, the social interaction test and the elevated plus-maze. In addition, because this has not previously been studied, the time-course of nicotine's effects on the plus-maze was also examined in the isolated and socially grouped animals.

4.2 Materials and Methods

Animals

A total of 447 male Lister hooded rats (Charles River, Margate, Kent, UK) weighing between 250-300g were used. Rats were housed either in isolation or in social groups of five for 7 days prior to the start of behavioural testing. Isolation housed rats were housed singly in a cage, 45cm x 28cm x 20cm high. The group housed cages were 56cm x 38cm x 20 cm high. All the cages were in racks that allowed the rats to see, hear and smell other rats. Food and water were freely available to all the animals. The room in which animals were housed was lit with dim light and maintained at 22°C. Lights were on from 0700-1900 h.

Apparatus

The social interaction and elevated plus-maze tests were used in these experiments. See Chapters 2 and 3 for descriptions of these tests.

Drug

(-)-Nicotine hydrogen tartrate (Sigma, Poole, UK) was dissolved in distilled water, and doses of nicotine were calculated as the base. All drug injections were given subcutaneously (s.c.), in a volume of 1 ml/kg body weight. Control animals received equal volume injections of distilled water.

Procedure

Social Interaction

Each rat was placed individually in the test arena for a 5 min familiarisation trial on the day prior to the social interaction test. Rats were allocated to test partners on the basis of weight, so they did not differ by more than 10g. For the group-housed rats, the test partner was always taken from a different cage. After testing, the rats were returned to their home cages, so that group-housed animals always remained in a group of at least four. In the main study, 48 pairs of singly housed rats and 42 pairs of group-housed rats were randomly allocated among the 6 drug groups (vehicle or (-)-nicotine 0.025, 0.05, 0.1, 0.25 or 0.45mg/kg; s.c.). On the basis of the results, a further 21 pairs of animals were housed in social groups for 7 days and then tested with vehicle or (-)-nicotine (0.01 or 1mg/kg; s.c.). In all experiments, both members of a test pair received the same dose of nicotine on the test day. On the test days, 30 min after injection, pairs of rats were placed in the centre of the arena and their behaviour scored for 4.5 min from a monitor in the adjacent room, by an observer with no knowledge of the drug treatment. The scores that were analysed were the total times spent interacting

by each pair of rats (i.e. a single score for each pair of rats). At the end of the trial any faeces were removed and the arena wiped with a damp cloth. Rats were tested between 0900-1300h.

Elevated Plus-maze

For the plus-maze experiments, 119 singly housed rats and 106 group-housed rats were randomly allocated among the 5 drug groups (vehicle or nicotine 0.05, 0.1, 0.25 or 0.45mg/kg s.c.). On test days, the rats receiving each drug treatment were further sub-divided into three groups, and were tested either 5, 30 or 60 minutes after their injection. Each rat was placed individually in the central square of the plus maze facing the closed arm, and its behaviour was scored for 5 min by an observer blind to the drug treatment. The number of entries onto the open and closed arms, and the time spent on the open arms, closed arms and in the central square was scored. Testing took place between 0900 and 1300 h in an order randomised for drug treatment. The arena was wiped with a damp tissue after each trial. After testing each rat was returned to its home cage.

Statistics

Data for the dose-response to nicotine in the social interaction test were analysed by a two-factor parametric analysis of variance (Factor 1, housing; Factor 2, drug treatment). A significant housing x drug interaction would show that the housing condition significantly influenced the drug effect. The data for the additional doses investigated only in the group-housed rats were analysed in a one-way analysis of

variance. The plus-maze data were analysed by a three-factor analysis of variance (Factor 1, housing; Factor 2, drug treatment; Factor 3, pre-treatment time). After the analyses of variance, comparisons between individual groups were made with Fisher's post-hoc tests and it is the significances of these that are shown in the figures and tables. A subsidiary analysis was conducted in order to determine whether the order of removal from the group influenced the scores in the two tests. The scores of the first and last rats to be removed from each cage were compared in all the groups in which nicotine had no effect. Thus, in the social interaction test the comparison included all groups, except for 0.025 mg/kg nicotine, and in the plus-maze the comparison included all the groups tested at the 60 min time-point.

4.3 Results

Social Interaction

Overall, the group-housed rats spent significantly less time in social interaction than did those that were singly housed [$F(1,78)=188.6$, $p<0.00001$]. There were dose-dependent effects of nicotine, but these varied significantly in the two housing conditions [housing x drug interaction, $F(5,78)=18.7$, $p<0.00001$]. In the singly-housed rats, nicotine (0.05, 0.1, 0.25 mg/kg) significantly increased social interaction, whereas 0.45 mg/kg significantly decreased it, see Figure 4.1. In comparison, in the group-housed rats, only 0.025 mg/kg nicotine significantly increased social interaction, see Figure 4.1. In the group-housed rats, the lowest dose of nicotine (0.01 mg/kg) non-significantly increased, and the highest dose (1 mg/kg) significantly decreased, social interaction (see Table 4.1).

Table 4.1 Mean (± sem) time (s) spent in social interaction (s) and locomotor activity (beam breaks) of rats housed in social groups of 5 for 7 days and then tested 30 min after injections of vehicle or (-) nicotine (0.01 or 1mg/kg s.c.). * $p< 0.05$, **$p<0.001$ compared with vehicle control group.

Drug Treatment (mg/kg)	Social Interaction (s)	Locomotor Activity
Vehicle	31.9 ± 5.8	243 ± 23.9
0.01	45.3 ± 5.7	206 ± 16.7
1	13.2 ± 1.5*	99 ± 17.5**

Overall, the group-housed rats had lower locomotor activity than the singly housed rats [$F(1,78) = 7.5$, $p<0.01$). Nicotine produced a dose-related decrease in locomotor activity [$F(5,78) = 2.8$, $p< 0.05$), that reached significance in both housing conditions at the 0.45 mg/kg dose and in the singly housed rats also at the 0.25 mg/kg dose (Table 4.2). There was no significant housing x nicotine interaction on locomotor activity [$F<1.0$].

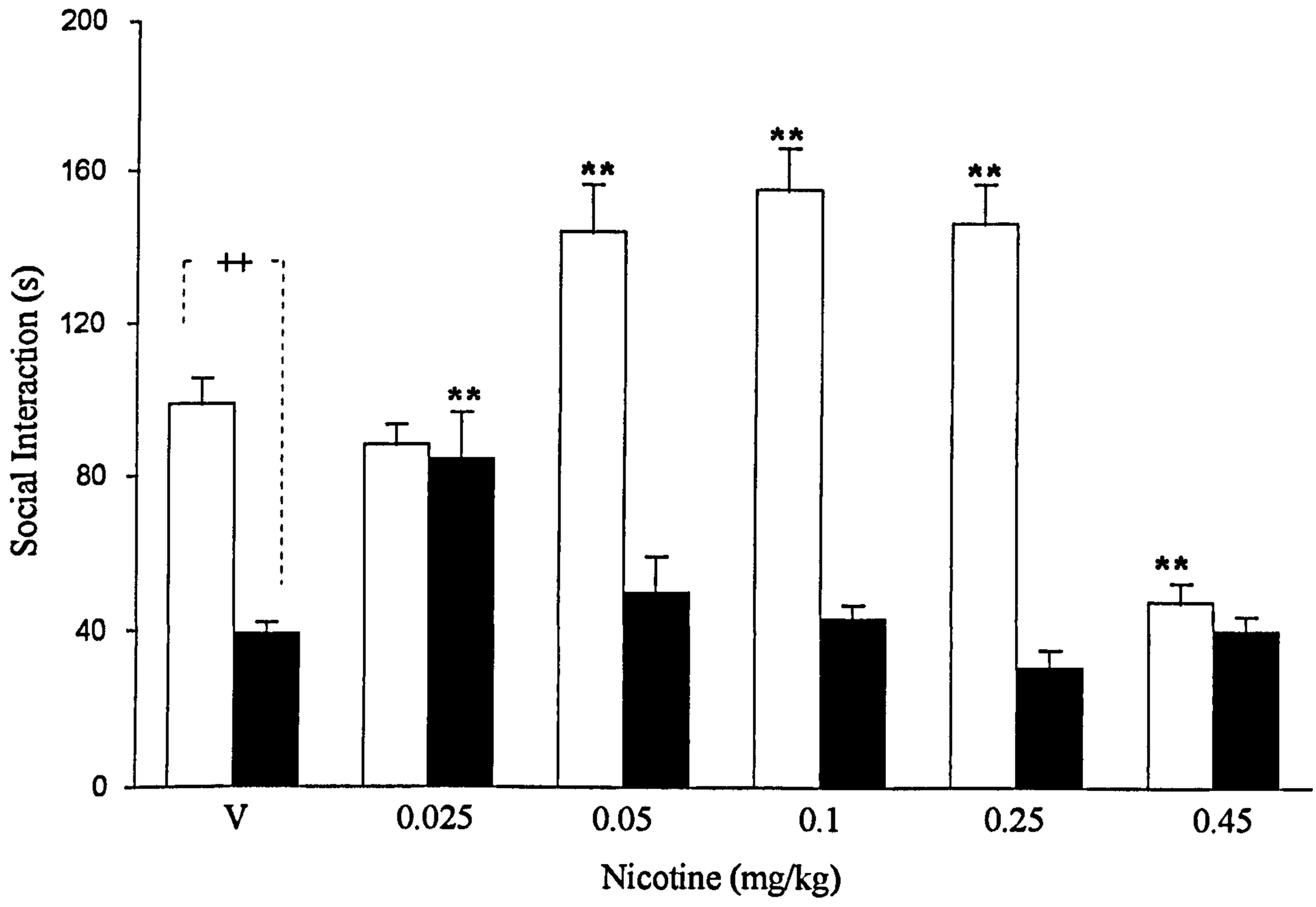

Figure 4.1 Mean (± sem) time (s) spent in social interaction by rats housed singly (clear bars) or in social groups of 5 (black bars) for 7 days and then tested 30 min after injection with vehicle or (-) nicotine (0.025-0.45mg/kg s.c.). **p<0.01 compared with appropriate vehicle control group. ++p<0.01 comparing vehicle-injected singly and group housed animals.

Table 4.2 Mean (± sem) locomotor activity (beam breaks) of rats housed singly or in social groups of 5 for 7 days and then tested 30 min after injection with vehicle or (-) nicotine (0.025-0.45mg/kg s.c.). *p<0.05 compared with vehicle control group

Drug Treatment (mg/kg)	Locomotor Activity	
	Single Housing	Group Housing
Vehicle	302 ± 19.6	266 ± 24.3
0.025	255 ± 14.6	241 ± 6.7
0.05	251 ± 21.1	203 ± 11.4
0.1	258 ± 21.1	219 ± 12.5
0.25	226 ± 18.7*	193 ± 40.0*
0.45	223 ± 31.2*	204 ± 21.9

Elevated Plus-maze

In the elevated plus-maze there was no overall effect of the housing conditions on any of the measures [F<1.4 in all cases]. However, the effects of nicotine on the measures of anxiety depended on both the housing conditions and the pre-treatment time [drug x housing x pre-treatment time, $F(8,195) = 2.4$, $p<0.05$ for % time on open arms and $F(8,195) = 2.6$, $p<0.01$ for % open arm entries). It can be seen from Figure 4.2 and Table 4.3 that in both singly and group-housed rats the doses 0.1 – 0.45 mg/kg nicotine significantly decreased the percentage of time spent on the open arms and the percentage of open arm entries. However, these anxiogenic effects of nicotine were time-related and in the group housed animals were more evident at the 5 and 30 min

times of testing, whereas in the singly housed animals they were significant at 30 and 60 min. Neither nicotine nor the housing conditions significantly affected the number of closed arm entries (see Table 4.3) and therefore nicotine had a specific anxiogenic effect in this test.

Effect of order of removal from the group

It can be seen from Table 4.4 that there was no difference in the time spent in social interaction or in the locomotor scores between the rats removed first from each cage and those removed last. However, in the elevated plus-maze the rats removed first showed an anxiogenic effect, indicated by decreased % of open arm entries [$F(1,13)=9.8$, $p<0.01$] and % time in the open arms [$F(1,13)= 5.5$, $p<0.05$] , see Table 4.4. There was no effect on the number of closed arm entries.

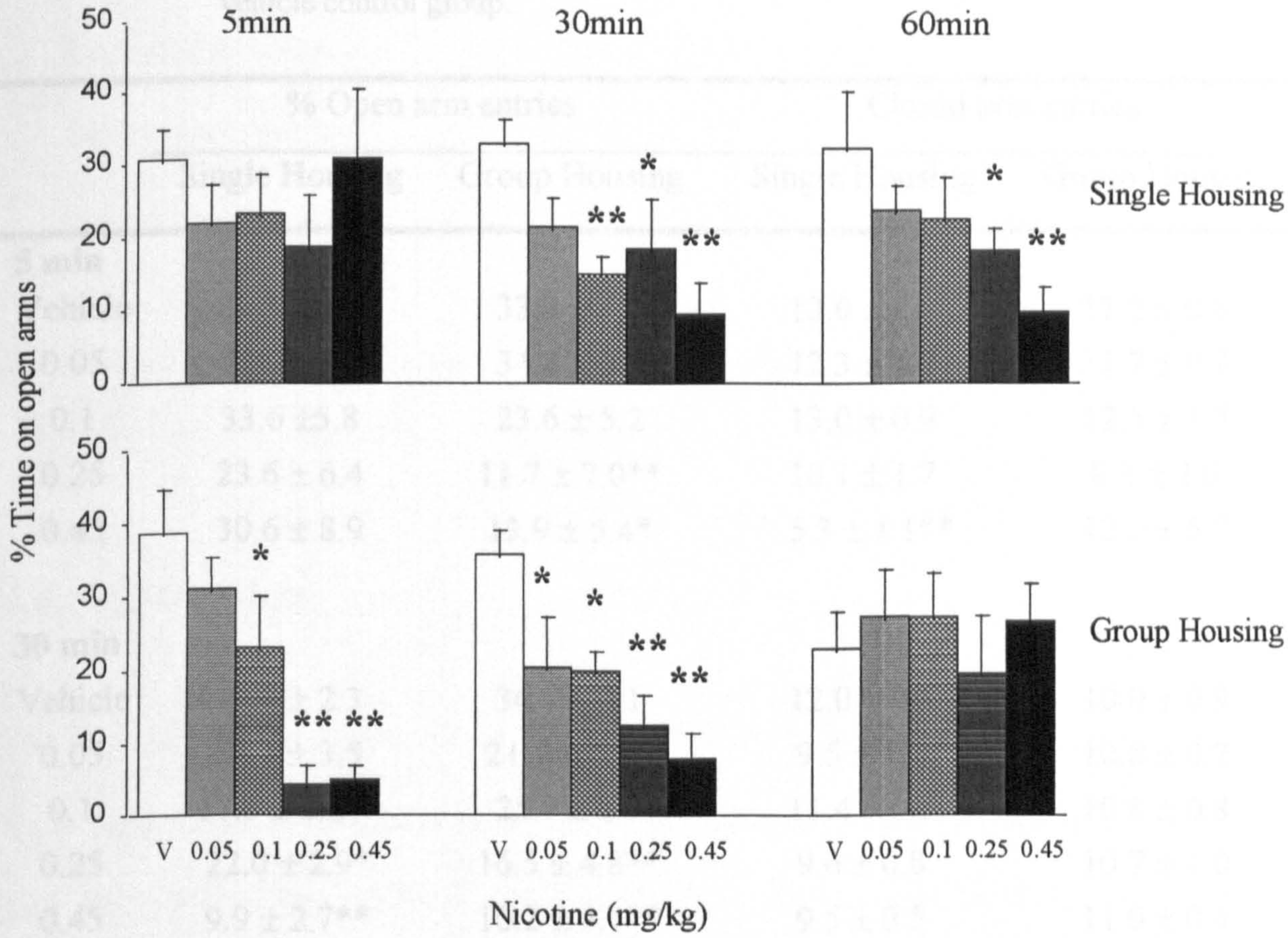

Figure 4.2 Mean (± sem) % time spent on the open arms of the elevated plus maze by rats housed singly or in social groups of 5 for 7 days and then tested 5, 30 or 60 min after injection with vehicle or (-) nicotine (0.05-0.45mg/kg). *p<0.05 and **p<0.001 compared with appropriate vehicle control group.

Table 4.3 Mean (± sem) % entries onto open arms and number of closed arm entries on the elevated plus maze in rats housed singly or in social groups of 5 for 7 days and then tested 5, 30 or 60 min after injection with vehicle or (-) nicotine (0.05-0.45mg/kg, s.c.). *p<0.05 and **p<0.001 compared with appropriate vehicle control group.

	% Open arm entries		Closed arm entries	
	Single Housing	Group Housing	Single Housing	Group Housing
5 min				
Vehicle	29.1 ± 1.8	33.9 ± 4.8	13.0 ± 2.8	11.2 ± 0.8
0.05	22.2 ±2.8	34.2 ± 2.4	12.3 ± 1.2	11.7 ± 0.9
0.1	33.6 ±5.8	23.6 ± 5.2	13.0 ± 0.9	12.5 ± 1.7
0.25	23.6 ± 6.4	11.7 ± 7.0**	10.1 ± 1.7	8.8 ± 2.6
0.45	30.6 ± 8.9	13.9 ± 5.4*	5.3 ± 1.1**	12.0 ± 5.7
30 min				
Vehicle	32.0 ± 2.3	36.7 ± 3.1	12.0 ± 0.5	10.0 ± 0.9
0.05	33.1 ± 3.5	21.5 ± 6.1*	9.5 ± 1.1	10.8 ± 0.7
0.1	17.6 ± 2.2**	25.7 ± 2.7	11.4 ± 0.5	10.8 ± 0.8
0.25	22.0 ± 2.9*	16.5 ± 4.8**	9.6 ± 0.8	10.7 ± 1.0
0.45	9.9 ± 2.7**	10.2 ± 4.1**	9.5 ± 0.5	11.0 ± 0.4
60 min				
Vehicle	33.5 ± 5.3	26.2 ± 5.2	10.0 ± 1.0	12.8 ± 0.8
0.05	28.5 ± 2.0	26.4 ± 4.4	10.6 ± 0.5	12.7 ± 0.4
0.1	19.8 ± 3.2*	25.2 ± 4.4	12.5 ± 0.7	13.7 ± 0.4
0.25	22.9 ± 3.5	20.8 ± 5.0	13.8 ± 1.2**	12.8 ± 1.1
0.45	12.9 ± 4.2**	25.2 ± 3.8	10.8 ± 0.6	12.1 ± 0.6

Table 4.4 Mean (± sem) time spent in social interaction (s), locomotor activity (beam breaks) % open arm entries, % time in the open arms and closed arm entries by rats removed first or 5th from the group cages. ** p<0.01, * p <0.05.

Order of removal	1st	5th
Social Interaction (s)	50.0 ± 5.9	40.9 ± 6.5
Locomotor Activity	209.3 ± 21.6	223.3 ± 22.6
% open arm entries	17.0 ± 3.1**	29.3 ± 2.2
% time on open arms	15.5 ± 3.1*	29.7 ± 5.4
Closed arm entries	13.6 ± 0.5	12.0 ± 0.8

4.4 Discussion

The increased social interaction in the singly housed rats confirmed earlier findings on the effects of social isolation in adulthood (Varlinskaya et al, 1999; Niesink & Van Ree, 1982; File & Pope, 1974a) and since weaning (Wongwitdecha & Marsden, 1996). This increased interaction is the main reason for routinely using singly housed rats in this test. However, this test has been used in group-housed rats (Costall et al, 1993; Dunn et al, 1991; Jones et al, 1988) and it is possible to detect anxiolytic effects of benzodiazepines in both housing conditions (Dunn et al, 1991; Gardner & Guy, 1984; File, 1980; File & Hyde, 1978). The present study showed that an anxiolytic effect of nicotine can be detected in both group and singly-housed rats, but it was manifested at a much wider dose-range in the singly-housed rats. This is perhaps surprising, since in general it is easier to detect increases in behaviour when baseline scores are low (e.g.

Crawley & Davis, 1982). Rate-dependent effects of nicotine have been detected in a wide range of behaviour, including its reinforcing effects (Perkins, 1999). Thus, it would seem to be the stress, or some other effect, of social isolation, rather than the baseline scores per se, that was enhancing the anxiolytic effects of nicotine in this test. Interestingly, the anxiogenic effects of nicotine were also enhanced in the isolated rats, which perhaps argues against an interpretation simply in terms of social isolation acting as a stressor.

The results also showed that short-term social isolation in adult rats enhanced locomotor activity, as has been shown previously for rats reared in social isolation (Hall et al, 1998; Smith et al, 1997). This raises the possibility that the increased activity is a response to the current housing conditions and not necessarily a consequence of the long-term effects of isolation rearing. However, the current housing conditions did not modify nicotine's effects on locomotor activity, whereas isolation since weaning did enhance the locomotor stimulant effects of amphetamine and ethanol (Hall et al, 1998; Smith et al, 1997).

In contrast to the social interaction test, the housing conditions did not modify behaviour in the elevated plus-maze, as has been previously found in mice (Rodgers & Cole, 1993). These results are also in general agreement with previous studies in which other test condition manipulations, such as extra-maze cues and lighting, were found not to influence baseline scores (Rodgers et al, 1997; Becker & Grecksh, 1996; Falter et al, 1992; Baldwin & File, 1986; Pellow et al, 1985). It is therefore even more

striking that the order of removal from the group did seem to affect behaviour, with the rats that were removed first having scores in the plus-maze that indicated increased anxiety. No such effects were found in a study on the effects of cohort removal in mice, but the mice were sequentially removed and not replaced in the group cage after testing (Rodgers et al, 1994). It is possible that the effects observed in this study were caused, not by cohort removal, but by the initial disturbance to the cage, to which the rats habituated after several disturbances. Further studies are clearly needed in this area. Another contrast between the two tests of anxiety was that the housing conditions did not influence the dose-response to nicotine in the plus-maze and only anxiogenic effects were detected in this test. The housing conditions did, however, alter the time at which nicotine's effects were detected, with the effects being delayed in the singly housed rats. Previously, rats reared in isolation have shown an altered time course of amphetamine-induced locomotor activity (Jones et al, 1992b). The shift in the time course in the present study does not appear to be dependent on baseline differences between singly and group housed rats. There is convincing evidence that the anxiogenic effects of nicotine in the elevated plus-maze are primarily mediated by 5-HT_{1A} receptors in the lateral septum (Cheeta et al, 2000b). The present results therefore indicate possible alteration of serotonergic dependent function of the lateral septum following isolation housing of rats. However, it is also possible that isolation housing altered the pharmacokinetic effects of nicotine, and that the later onset of nicotine's anxiogenic effect was due to changes in nicotine absorption and metabolism, although as yet this still has to be determined.

Several factor analysis studies have provided evidence that different animal tests of anxiety are reflecting different underlying factors, and hence may be modelling different anxiety disorders (Flaherty et al, 1998; Ramos et al, 1997; Belzung & Le Pape, 1994; File, 1992). Inbred rat lines have showed different effects in different tests of anxiety (Chaouloff et al, 1994) and further differences between these tests have been shown in the effects of brain lesions and central drug administration (Menard & Treit, 1999). Differential effects have also been found following systemically administered drugs (Fernandez-Guasti et al, 1999; Treit et al, 1993) and after the stress of inescapable shock (Steenbergen et al, 1990).

The differences found between the social interaction test and the elevated plus-maze in the effects of nicotine. Thus, in Lister hooded rats and in the test conditions used, although low doses of nicotine were anxiolytic in the social interaction test, only anxiogenic effects have been found in the plus-maze (this experiment and Ouagazzal et al, 1999a; File et al, 1998). It is perhaps most striking that, in singly housed rats, nicotine (0.1 mg/kg 30 min after injection) had an anxiolytic action in the social interaction test, yet an anxiogenic effect in the plus-maze (Kenny et al, 2000b; Ouagazzal et al, 1999a). There was no dose of nicotine that had an anxiolytic effect in the plus-maze after acute administration. The lowest dose investigated in the present study was 0.025 mg/kg, but Ouagazzal et al (1999a) also found no effects with doses as low as 0.01mg/kg. A further difference seems to lie in the brain regions mediating nicotine's anxiogenic effects in the two tests, which implies that different brain regions may be activated by the two tests. Thus, while both the dorsal hippocampus and the

lateral septum mediate the anxiogenic effects of nicotine in the social interaction test, only the latter structure mediates effects in the plus-maze (Cheeta et al, 2000b; Kenny et al, 2000b; Ouagazzal et al., 1999a, b). In all cases the anxiogenic effects were antagonised by co-administration of the $5HT_{1A}$ antagonist, WAY 100,635 (Cheeta et al., 2000b; Kenny et al., 2000b). Since the group-housed rats did not show any anxiogenic effects to nicotine in the social interaction test, this implies that the housing conditions might have modified the nicotinic cholinergic and/or serotonergic systems in these brain regions.

In conclusion, the results showed that even a few days of social isolation in adulthood can profoundly affect baseline responses in tests of anxiety and responses to nicotine. Singly housed rats have also shown greater physiological reactions to stress than did group-housed rats (Ruis et al, 1999; Baldwin et al, 1995). However, an increased stress response to the behavioural tests is an insufficient explanation for the pattern of results found with nicotine in this study.

CHAPTER 5

Nicotine self-administration and withdrawal: modulation of anxiety in the social interaction test in rats

5.1 Introduction

Nicotine is known to produce a wider rang of behavioural effects (for review see Stolerman et al., 1995) but one of the most striking behavioural properties is its reinforcing effects which results in tobacco addiction in smokers. Nicotine is one of the most widely abused substances in the world and in Great Britain it is estimated that 28% of men and 25% of women smoke (Royal College of Physicians, 2000). Drug self-administration studies represent the most established paradigm available to investigate the reinforcing properties of abused substances in animals. Corrigall and Coen (1989) developed reliable schedules of intravenous nicotine self-administration in rats that has been replicated by other groups (Donny et al., 1995; Tessari et al., 1995).

There is considerable evidence that the behavioural effects of nicotine change after repeated injections. In the social interaction test in rats, after 7 daily injections, tolerance developed to both the anxiolytic and anxiogenic effects of nicotine (0.1 mg/kg) and there was an anxiogenic response 72 h after withdrawal from 7 and 14 days of treatment (Chapter 2). In order to examine the possible role of anxiety in nicotine self-administration the purpose of the present experiment was to determine the changes in anxiety that occur when rats are self-administering nicotine and when they are

withdrawn from nicotine after 4 weeks of self-administration. Intravenous self-administration of nicotine in rats is considered a useful model of nicotine dependence (Corrigall, 1999; Rose & Corrigall, 1997). In this study, rats that had been self-administering nicotine for 4 weeks were tested in the social interaction test of anxiety after their normal daily nicotine session and again after 24 and 72 h of withdrawal. In testing the effects of withdrawal from nicotine the animals were tested under extinction conditions, i.e. no nicotine reinforcements were delivered. In order to assess the importance of nicotine-associated cues, the conditioned stimuli were still presented during the extinction sessions and the performance of animals exposed to these cues was compared with a group that was tested immediately after removal from the home cage.

5.2 Materials and Methods

Animals

Male Wistar rats (Charles River, Germany) were individually housed in a temperature-controlled environment with lights (<50 scotopic lux) on from 0600-1800 h. During the experiments, water was continuously available and animals were maintained at a constant body weight of 240-260g (85% of their ad libitum body weight).

Apparatus

Operant Chamber

The self-administration sessions were conducted in sixteen operant chambers (Coulbourn Instruments, Lehigh Valley, USA) encased in sound-insulated cubicles, equipped with ventilation fans (Ugo Basile, Comerio, Italy). Each chamber was equipped with two levers, symmetrically centred on the front panel, located 12.5 cm apart, 2 cm above the grid floor. The food magazine was situated in an opening in the panel between the two levers, 1 cm above the floor. A 2 W white house light was located 26 cm above the food magazine and activated throughout the entire session. Presses on the right lever ("active lever pressing"), corresponding to the Fixed Ratio values required by the schedules of reinforcement, produced delivery of 45 mg food pellets (Bioserv, Frenchtown NJ, USA) or the activation of the infusion pump, except during withdrawal conditions. Delivery of reinforcement was signalled by the 1 s illumination of a 4 W white stimulus light located in the same hole of the food magazine and by the 1 s sounding of a Sonalert device (2.9 Hz, 60 dB). Drug or vehicle solutions were administered via the infusion pump (Model A-99Z, Razel Scientific Instruments inc., Connecticut, U.S.A.) at a volume of 0.022 ml during a 1 s period. Presses on the left lever did not have any consequence. Lever presses, food pellet and infusion deliveries were recorded. Data acquisition and schedule parameters were controlled by a Med-PC software (Med Associated, Georgia VT, USA) running on two Compaq microcomputers interfaced with the chambers via interface modules (Med Associated, Georgia VT, USA).

Social Interaction Test Arena

See Chapter 2 for description.

Training to lever-press for food reinforcement

Following a 24 h deprivation period, rats were trained to press the right lever for food as a reinforcer. They were trained to a final Fixed-Ratio = 2 (FR 2) schedule with a Time-out of 60 s (TO 60s). Each session lasted until the rat had received 100 pellets of food or was terminated after a maximum of 2 h. Once they reached this criterion, the rats underwent surgery.

Surgery

The surgical procedure described by Lane et al., (1992) was used with minor modifications. Rats were anaesthetised with a mixture of chlordiazepoxide (9 mg/kg; F.I.S., Alte Ceccato, Italy) and ketamine (50 mg/kg; Ketavet®100, Farmaceutici Gellini S.p.A, Aprilia, Italy), 1ml/kg IP (5 min pretreatment with atropine sulphate 1 mg/kg IP; Sigma, St. Louis MO). They were then were implanted with a Silastic catheter (inner diameter 0.012 in, outer diameter 0.025 in; Dow Corning, Michigan, USA) in the right jugular vein. The free end of the catheter was connected to a connector consisting of a modified C313G cannula assembly (Plastic One, Roanoke VA, USA) and the resulting unit was mounted to the skull with dental acrylic cement and fixed via three stainless steel screws. Animals were injected IV with 0.1 ml of a solution containing 1 IU/ml heparin (Liquemin®, Roche S.p.A., Milano, Italy) and 65

mg/ml ticarcillin plus clavulanate (SmithKline Beecham, Milano, Italy), This treatment was repeated every 12h for 7 days after surgery (period of recovery).

Nicotine Self-administration Training

After the period of recovery, thirty-two rats were randomly allocated to self-administer either saline (n=15) or nicotine (n=17; 0.03 mg/kg/infusion; Sigma, St. Louis MO, USA). Nicotine self-administration was initiated on an FR 1 schedule of reinforcement with a TO 60 s after each infusion. During the time-out period, responses were recorded but did not lead to drug delivery. For the FR1 schedule of reinforcement, each session lasted until the rat had received 25 infusions of nicotine or was terminated after a maximum of 3h. If the animals met the criterion of 25 infusions within the end of the daily session, the FR value was increased to FR 2 the following day. For the FR2 schedule of reinforcement, each session lasted until the rat had received 15 infusions of nicotine or was terminated after a maximum of 2 h. After about two weeks of training all rats were receiving 15 infusions of nicotine, and this was the case on the first social interaction test day, which took place after 4 weeks of self-administration. The control rats that were receiving saline infusions had their first session on the FR1 schedule of reinforcement but, were placed on an FR2 schedule for the subsequent training sessions. These rats had the same number of training sessions as the rats self-administering nicotine. Each control rat was paired with a rat self-administering nicotine, and the amount of time the control rat spent in the operant chamber was determined by the time that its partner took to receive its 15 infusions of nicotine.

Drugs

All drugs were dissolved in heparinized saline (0.09% NaCl + 0.5 UI/ml heparin) and the pH was adjusted to 7.4 with NaOH. Nicotine doses are expressed as mg of free base/kg of body weight per infusion.

Social Interaction Test Procedure

The day before their first social interaction test, the rats received their usual daily self-administration session in the morning and in the afternoon they were familiarised with the test arena. Each rat was placed singly in the brightly lit arena, for a 10 min familiarisation trial. On each test day, each experimental rat was placed in the social interaction test with an unoperated partner and tested for 4.5 min. At the end of the test, the rats and any faecal boluses were removed and the arena wiped with a damp cloth. All animals were tested in an order randomised for drug treatment, between 0900 and 1400 h.

On the first social interaction test day, each rat received its usual daily self-administration session and 5 min after it had received its 15^{th} infusion of nicotine it was placed in the social interaction test arena. Rats that were receiving saline were taken out of the operant chamber at a time corresponding to a nicotine animal. All the animals were then tested again 24 and 72 h later, without receiving any further nicotine or saline infusions. At both these time-points, the animals were either taken directly

from their home cages (saline, n=7; nicotine, n=9) or were tested 5 min after a 30 min extinction session in the operant chamber (saline, n=8; nicotine, n=8). During the 30 min extinction session, responding on the 'active lever' lever caused the presentation of the conditioned stimuli (FR1), but no delivery of nicotine or saline.

Statistics

The scores of the chronically treated rats were compared by one-way ANOVA. The scores from the tests in withdrawal conditions were analysed by two-way ANOVAs with chronic drug treatment (vehicle or nicotine) as one factor and experience prior to test (directly from the home cage or exposure to the operant chamber) as the other factor. Differences between individual groups were then assessed by Fisher's post-hoc tests, and it is these that are presented in Figure 5.1 and Table 5.11. Pearson's product-moment correlation coefficient was calculated for the social interaction score and the time taken to receive 15 nicotine infusions on the test day.

5.3 Results

The rats that had been self-administering nicotine spent significantly less time in social interaction than the group that had been self-administering vehicle, [$F(1,31)=21.6$, $p<0.01$; Figure 5.1]. The chronic nicotine self-administration did not result in any decreases of locomotor activity, see Table 5.1. Thus, this pattern of results indicates a specific anxiogenic effect of the chronically self-administered nicotine. For the nicotine self-administration group there was no correlation between the time taken to

receive 15 nicotine infusions on the test day and the social interaction score (r=-0.18). Thus, the anxiogenic effect was not dependent on the rate at which the nicotine dose was received.

The rats that were withdrawn for 24 and 72 h from chronic nicotine administration, did not differ from their saline control group in the time they spent in social interaction (see Figure 5.1) or in their locomotor activity (see Table 5.1). Thus, there was no evidence from these measures of a withdrawal response in the animals that had been self-administering nicotine for 4 weeks. However, there were significant effects that resulted simply from exposure to the operant chamber immediately prior to the social interaction test, regardless of drug treatment. The rats exposed to the chamber on the first extinction day spent significantly less time in social interaction than did those that were tested directly from their home-cages [$F(1,28)=11.9$, $p<0.005$]. This can be seen in Figure 5.1 by comparing the 24 h nicotine group tested straight from the home cage with the nicotine 24 h group that had prior exposure to the operant chamber, and by comparing the two saline 24 h groups. The rats exposed to the operant chamber on the second extinction day (72 h groups in Figure 5.1) also spent less time in social interaction than did those tested directly from the home-cage, but this no longer reached significance [$F(1,28)=2.5$]. There were no changes in locomotor activity as a result of exposure to the operant chamber, at either of the time intervals tested (see Table 5.1).

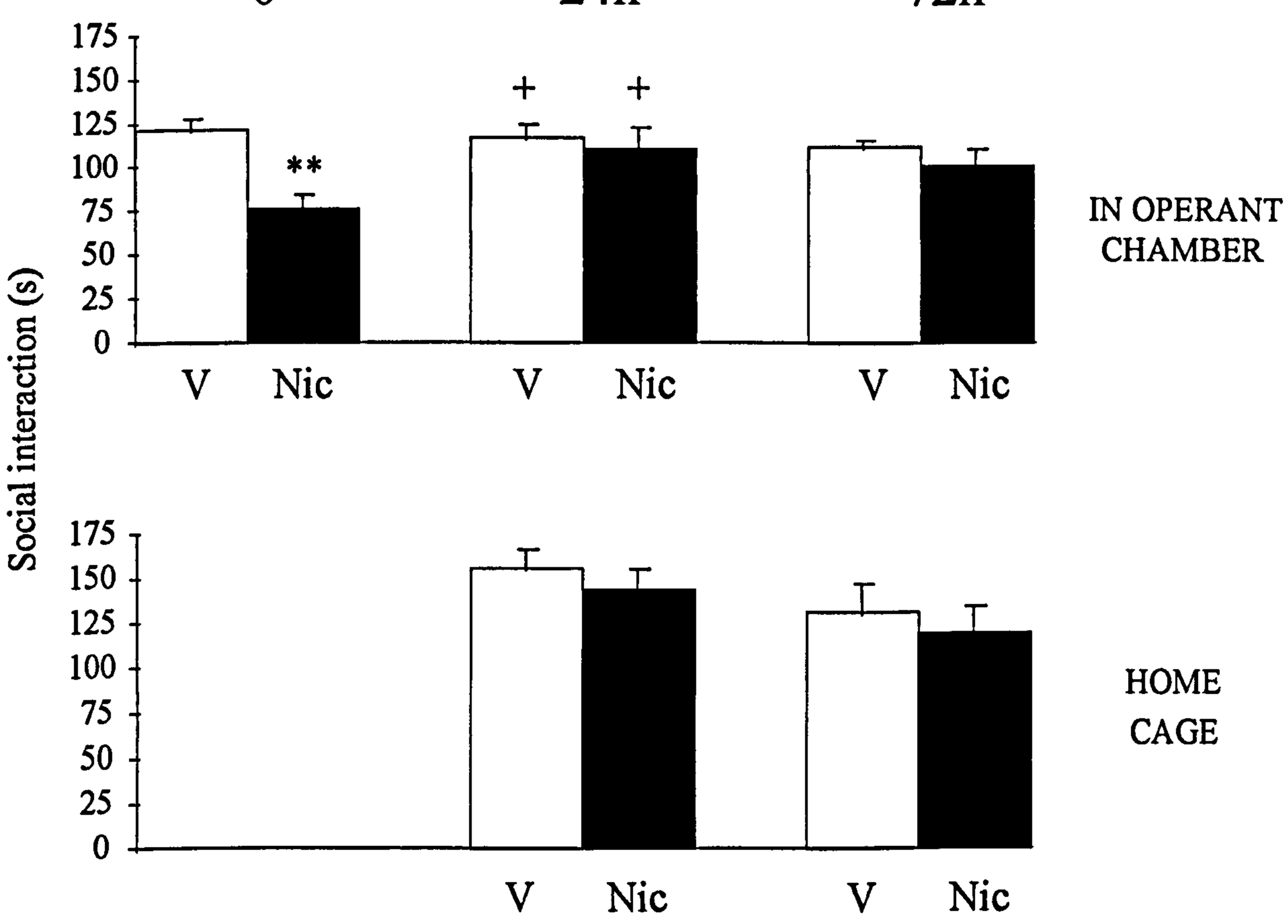

Figure 5.1 Mean (± sem) time spent in social interaction by rats tested 5min (0h), 24h and 72 h after saline (V) or 4 weeks of nicotine (0.45 mg/kg/session; Nic). The upper panel shows the scores for rats tested 5-min after a session in the operant chamber; the lower panels shows scores for rats tested directly after removal from the home-cage. All tests were conducted in the high light familiar (HF) test condition. **p<0.01 compared with appropriate vehicle control, +p<0.01 compared with corresponding group tested directly from the home cage.

Table 5.1 Mean (± sem) locomotor activity (number of line crossings) made by rats tested 5min (0h), 24 and 72 h after saline (V) or 4 weeks of nicotine (0.45 mg/kg/session; Nic). On the first test day (0h), the animals were tested after their normal daily session in the operant chamber. For the 2 withdrawal time-points the animals were either taken directly from the home cage (Home Cage) or were tested 5 min after a 30 min session in the operant chamber (Operant Chamber). All tests were conducted in the high light familiar (HF) test condition.

	0h		24h		72h	
	V	Nic	V	Nic	V	Nic
Operant Chamber	114.7 ± 9.0	163.2 ± 9.3	129.6 ± 12.7	115.7 ± 17.0	126.9 ± 11.9	137.0 ± 16.5
Home Cage	-	-	116.3 ± 14.6	139.2 ± 7.7	141.1 ± 17.3	147.0 ± 14.5

5.4 Discussion

Rats that had been self-administering nicotine for 4 weeks showed a decrease in the time spent in social interaction when tested straight after their self-administration session. This can be interpreted as an anxiogenic effect, since it was not accompanied by any reduction in locomotor activity. The results therefore show that chronic self-administration of nicotine can be accompanied by an anxiogenic effect, as detected in the social interaction test. This suggests that either this dose of nicotine is sufficiently rewarding to overcome the adverse consequences of increased anxiety, or that the relatively mild increase in anxiety contributes to the rewarding effects. At present it is impossible to distinguish between these two alternatives.

The anxiogenic effect of chronic nicotine that we found in this study is at variance with the widely held belief that nicotine is anxiolytic in smokers. There is considerable evidence that smoking alleviates the increased anxiety resulting from abstinence, but little evidence that it has an anxiolytic effect in non-deprived smokers (Parrott, 1999). Indeed, there is evidence that smokers experience higher levels of stress and anxiety than non-smokers (Jones & Parrott, 1997; West, 1992; Warburton et al, 1991). Adolescent smokers also report significantly higher levels of nervousness, stress and anxiety than age-matched non-smokers (Lloyd & Lucas, 1997; Wills, 1986; Mitic et al, 1985; Hirschman et al, 1984). It has generally been assumed that these increased anxiety levels are due to the periods of heightened anxiety during abstinence. The results of this study raise the possibility that chronic nicotine, even when self-administered, may itself have an anxiogenic effect. This may be related to the dose of nicotine and certainly after acute administration, high doses have anxiogenic effects in the social interaction test, whereas low doses have anxiolytic effects (File et al., 1998).

At the two time-points tested, there was no evidence of an anxiogenic effect resulting simply from nicotine withdrawal, whether or not the animals were exposed to the extinction conditions of the operant chamber. This is surprising, since following 7 and 14 days of sub-cutaneous administration of nicotine a significant decrease in social interaction was detected after 72 h of drug withdrawal (Chapter 2). However, there are several possible explanations for this result. One is that the wrong time-points had been chosen to detect such an effect following this particular dose-regime. The second is that withdrawal changes in anxiety were not detected because tolerance had not

developed to the anxiogenic effect of nicotine. The third is that these rats were accustomed to a 72 h period of abstinence every weekend, and that this may in some way have modified the withdrawal response.

An interesting side finding of this experiment was that exposure to the operant chamber produced an anxiogenic effect in all of the animals, regardless of their drug group. This effect was not therefore the result simply of removal of nicotine delivery and suggests that confinement to the test chamber generates a level of anxiety. The anxiogenic effect was observed in those tested at the 24 h time-point and since it was equal in both the nicotine and control groups it is clear that exposure to nicotine-associated cues was not generating the anxiety. At the 72 h time-point there was no longer a significant effect of exposure to the operant chamber on social interaction.

In conclusion, a main finding of this study is that increased anxiety when animals are withdrawn for 24 or 72 h from 4 weeks of nicotine self-administration is not contributing to self-administration in our test conditions. Furthermore, self-administration is maintained, not because of an anxiolytic effect of the self-administered nicotine, but despite (or because of) its anxiogenic effect. These results suggest that the increased anxiety and stress in smokers might be partially caused by persisting anxiogenic effects of nicotine and not solely due to those relating to withdrawal effects.

CHAPTER 6

Different treatment regimens and the development of tolerance to nicotine's anxiogenic effects

6.1 Introduction

The treatment regimen by which a drug is administered can be an important factor that influences behavioural effects, and is one that is often overlooked. It has been shown that chronic treatment with drugs such as the benzodiazepines, amphetamine and cocaine has differing effects on behaviour depending on the route by which it is administered (Fernandes et al., 1999 Koff et al., 1994; King et al., 1992; Ellison and Morrison, 1981). Tolerance to the behavioural effects of nicotine has been seen after daily subcutaneous, intraperitoneal or intravenous injections, constant infusion or intake in drinking water (Chapters 2 and 3; Sparks and Pauly, 1999; Marks et al., 1987, 1983; Clarke and Kumar, 1983 a, b; Stolerman et al., 1974, 1973), but at present there is little in the literature investigating the differential effects between these treatment regimens. A study by Morgan and Ellison (1987) showed opposing effects on body weight in female rats when the same dose of nicotine (11.2 mg/kg/day) was administered chronically by subcutaneous injection or pellet infusion. Marks et al. (1987) found that mice exposed to chronic nicotine (4 mg/kg/day over a 1h period) by discrete pulses (1-4 pulses/h) developed tolerance faster to the acute effects of nicotine on Y-maze activity than those receiving the same dose of nicotine by continuous

infusion. However, the up-regulation in [^{3}H]-nicotine binding was the same in the two treatment regimens.

Following 7 days of treatment with this low (0.1 mg/kg; s.c.) dose of nicotine, tolerance developed to both the anxiogenic and anxiolytic effects, and 72h after withdrawal from the nicotine treatment an anxiogenic effect was observed in the social interaction test (Chapter 2). However, in the previous chapter an anxiogenic effect was observed in the social interaction test in animals that had been self-administering nicotine (0.45 mg/kg/day, 5 days/week) for 4 weeks and were tested 5 min after their daily self-administration session. In these animals, there was no evidence of an anxiogenic withdrawal response either 24 or 72h after the last self-administration session (Chapter 5). Since the two studies differed in both the dose and treatment regimen, it is not possible to say whether tolerance did not develop because the animals were self-administering nicotine or because a higher dose was used.

Thus, the purpose of this study was to examine the effects on the development of tolerance to the anxiogenic effect of a high dose of nicotine using different treatment regimens. In all cases, rats received the same daily dose (0.45 mg/kg/day). One group of rats was passively administered i.v. doses of nicotine in the same pattern as that used for self-administration (15 infusions of 0.03 mg/kg, totalling 0.45 mg/kg/day, 5 days/week). Two groups received s.c. injections of 0.45 mg/kg/day, but one received 5 injections/week and the other received daily injections (7 injections/week). The final group received the same daily dose, but infused at a constant rate by an osmotic

minipump. The animals were tested in the social interaction 5 min after their normal daily nicotine injection or straight from the home cage (for the minipump group), and after 24 and 72h withdrawal.

In order to determine whether there was any evidence for the development of pharmacokinetic tolerance, plasma nicotine concentrations were determined by gas chromatography (Feryerabend & Russell, 1990).

6.2 Materials and Methods

Animals

Male Sprague-Dawley rats (Harlan Olac, Bicester, UK) were individually housed in the same room, maintained at 22°C, with lights (<50 lux) on from 0700 to 1900h. Food and water were freely available. At testing, the animals weighed between 300-375g.

Nicotine Treatment

Intravenous administration: The surgical procedure described by Lane et al., (1992) was used with minor modifications. Rats were anaesthetised by inhalation of 3 % isoflurane (May and Baker, Dagenham, Essex, UK) in oxygen and were then were implanted with a silastic catheter (inner diameter 0.012 in, outer diameter 0.025 in; Bio Pure Technology Ltd, Hampshire, UK) in the right jugular vein. The free end of the catheter was connected to a connector consisting of a modified C313G cannula assembly (Plastic Products, UK) and the resulting unit was mounted to the skull with

dental acrylic cement and fixed via three stainless steel screws. Animals were injected i.v. with 0.1 ml of a solution containing 1 IU/ml heparin (Monoparin®, CP Pharmaceuticals Ltd, Wrexham, UK). This treatment was repeated every 12h for 7 days after surgery (period of recovery). After the period of recovery, the animals received infusions of either vehicle or nicotine (0.03 mg/kg/infusion) every min for 15 min, so that the nicotine animals received a total of 0.45 mg/kg/day. The drug solutions were administered at a volume of 0.025ml during a 3 s period.

Subcutaneous injections: Animals received daily morning injections (5 or 7 days/week) of either nicotine (0.45 mg/kg) or vehicle. All injections were in a volume of 1 ml/kg.

Subcutaneous infusion: Rats were anaesthetised by inhalation of 3 % isoflurane (May and Baker, Dagenham, Essex, UK) in oxygen and osmotic minipumps (Alzet, USA) delivering 0.45 mg/kg/day nicotine were subcutaneously implanted in the dorsal thoracic area. Animals were monitored daily and the osmotic minipumps manipulated by hand within the subcutaneous pouch to reduce the amount of connective tissue growing around the pump that could impair infusion rate.

Apparatus

The social interaction test, see Chapter 2 for description.

Drug

For the i.v. injections, (-)-nicotine hydrogen tartrate (Sigma, Poole, UK) was dissolved in heparinized saline (0.09% NaCl + 0.5 UI/ml heparin) and for the s.c. injections and the minipumps it was dissolved in distilled water. Nicotine doses are expressed as mg of free base/kg of body weight.

Procedure

In order to familiarise rats with the social interaction test arena, each rat was placed singly in the test arena under high light for a 10 min familiarisation trial on the day prior to testing. For each experiment, animals were allocated to test partners on the basis of weight, such that members of a pair did not differ by more than 10g. On the test day, each experimental rat was placed together with its unoperated/uninjected partner in the test arena, 5 min after its daily injection or straight from the home cage (for the minipump groups). Social interaction was scored only for interaction that was initiated by the nicotine-treated animal, and was scored for 4.5 min by an observer blind to the drug treatment. All animals were tested in an order randomised for drug treatment, between 0900 and 1230h. At the end of each trial, any faecal boluses were removed from the test arena, which was cleaned with a damp cloth.

Experiment 1: i.v. administration of nicotine

Rats were randomly allocated to vehicle or nicotine (0.45 mg/kg) groups and within these they were allocated to be tested after an acute injection (vehicle, n=5; nicotine,

n=11), after 4 days of injections (vehicle, n=7; nicotine, n=8) or 4 weeks (vehicle, n=4; nicotine, n=4). Animals that had been treated for 4 weeks were then retested undrugged 24 and 72h later. The small group sizes in the 4 week treatment groups are due to blockade of indwelling catheters over this period.

Experiment 2a: s.c. injections

Rats were randomly allocated to the following groups: vehicle (n=10) and nicotine (0.45 mg/kg) treatment for 4 days (n=10) or 4 weeks (n=20). Half of each of these groups received either 5 (Monday - Friday) or 7 (every day) injections per week. Immediately after test, four animals from the 4 day nicotine group and four from each of the 4 week nicotine treated groups (5 and 7 days/week) were taken for determination of plasma nicotine levels. The remaining animals in the chronic nicotine treatment group were retested undrugged 24 and 72h later with their vehicle controls.

Experiment 2b: s.c. minipump

Rats were randomly allocated to the following osmotic minipump groups: vehicle (n=7) and 4 days (n=10) or 4 weeks (n=10) of nicotine (0.45 mg/kg/day). Immediately after test, four of the animals from each of the nicotine treated groups were taken for determination of plasma nicotine levels. The remaining animals in the 4 week treatment groups had their osmotic minipumps removed under anaesthesia and were then retested undrugged 24 and 72h later.

Experiment 2c: Determination of Plasma Nicotine Levels

To compare the plasma nicotine levels of the animals that had been treated for 4 days or 4 weeks with nicotine, animals were taken straight from testing, killed by decapitation and trunk blood was taken. The blood was centrifuged for determination of plasma levels of nicotine by gas chromatography, using nitrogen phosphorus detection with detection limit of 100 pg/ml using 100 µl of plasma (Feyerabend & Russell, 1990). Three of the blood samples became contaminated and were therefore excluded from statistical analysis.

Statistics

For each experiment, the scores were analysed by one-way ANOVA and comparisons between individual groups were then made with the Fisher's post-hoc tests. Because of the low numbers of animals that were tested after 4 weeks of i.v. nicotine, the scores were compared using Mann-Whitney U-tests (although for ease of comparison all the scores in Figure 1 are presented as means ± sem). The plasma concentrations of nicotine were also assessed by Mann-Whitney U-tests due to the low number of animals used.

6.3 Results

Tolerance and withdrawal after 4 weeks of i.v. nicotine

It can be seen from Figure 6.1 that an acute dose of nicotine significantly decreased the time spent in social interaction [$F(1,14)=56.3$, $p<0.00001$], indicating an anxiogenic

effect. There were still significant anxiogenic effects after 4 days [F(1,13)=,19.6 $p<0.001$] and 4 weeks (U=0, $p<0.05$) of nicotine treatment. Thus, although some tolerance appeared to have occurred it was not complete. There were no significant changes in locomotor activity [F(1,14)=0.5, F(1,13)=0.7 and U=4, respectively], see Figure 6.1.

The animals that were withdrawn for 24 and 72h from 4 weeks of nicotine administration did not differ from their saline control group in the time they spent in social interaction (U=8 and U=5, respectively), see Figure 6.1. However, the animals tested 24h after withdrawal from nicotine showed a significant increase in locomotor activity compared with saline controls (U=0, $p<0.05$), but this had disappeared by 72h (U=4), see Figure 6.1.

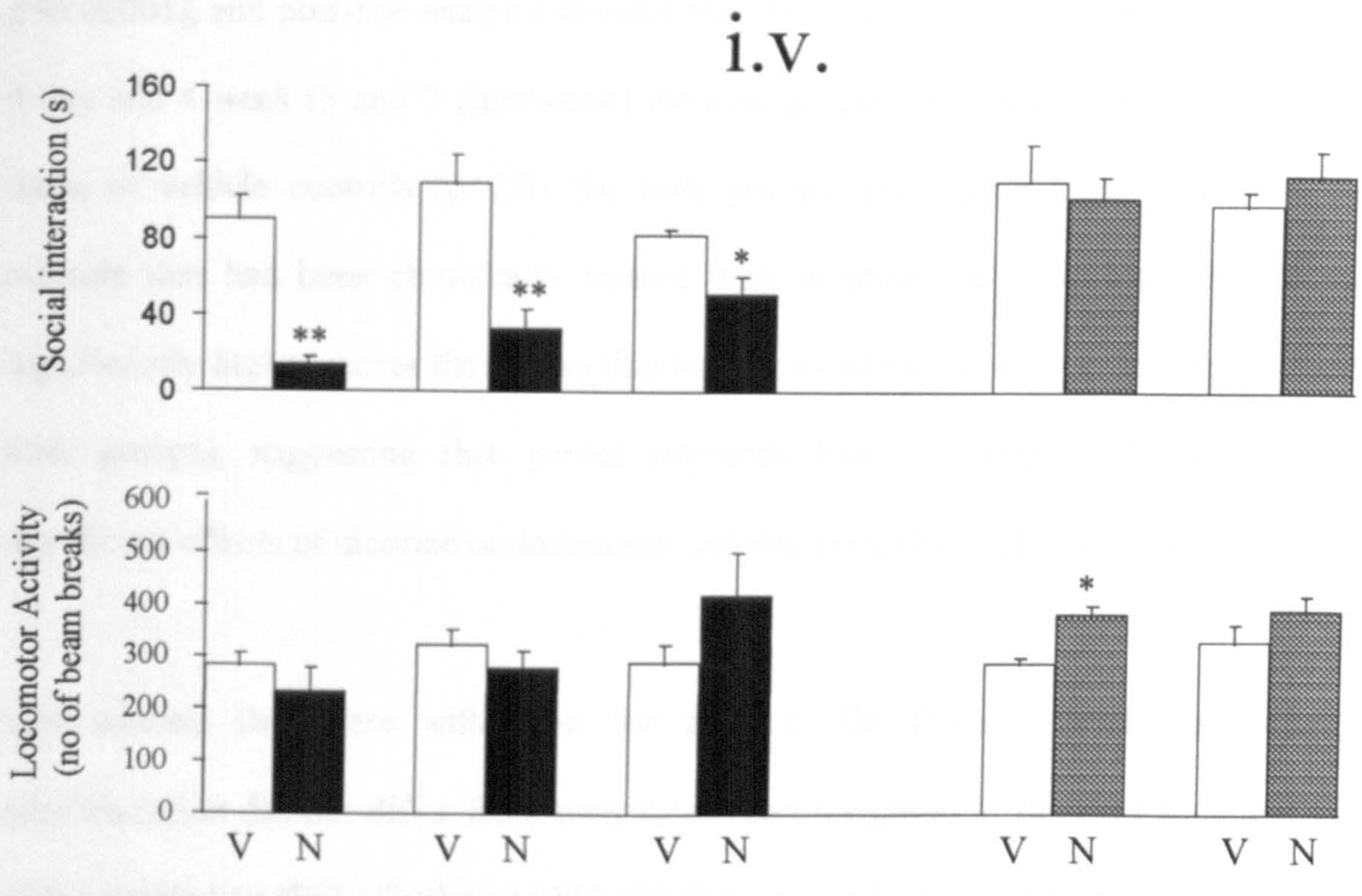

Figure 6.1 Mean (± sem) time (s) spent in social interaction (top panel) and locomotor activity (no of beam breaks; bottom panel) made by rats tested 5 min after acute (AC), 4 days (4D)or 4 weeks (4W, 5 injections/week) of intravenous vehicle or nicotine (0.45 mg/kg/day), and 24 and 72h after 4 weeks of nicotine treatment. Rats were tested in the high light familiar (HF) test condition. * $p<0.05$ and ** $p<0.01$ compared with the vehicle control.

Tolerance and withdrawal after 4 weeks of s.c. nicotine injections

In animals tested 5 min after a s.c. injection of nicotine (0.45 mg/kg) there was a significant effect of nicotine on the time spent in social interaction [$F(3,36)=15.3$, $p<0.00001$], and post-hoc analysis showed that this was due to the scores from both the 4 day and 4 week (5 and 7 days/week) nicotine groups being significantly lower than those of vehicle controls ($p<0.01$ for both groups; see Figure 6.2). However, the animals that had been chronically treated with nicotine for 5 or 7 days/week had significantly higher scores than those that had received nicotine for 4 days ($p<0.01$ for both groups), suggesting that partial tolerance had developed. There were no significant effects of nicotine on locomotor activity [$F(3,36)=2.2$], see Table 6.1.

The animals that were withdrawn for 24 and 72h from 4 weeks of nicotine administration did not differ from their saline control group in the time they spent in social interaction [$F(2,19)=0.4$ and $F(2,19)=0.9$, respectively; see Figure 6.2] or in their locomotor activity [$F(2,19)=1.1$ and $F(2,19)=1.3$, respectively], see Table 6.1.

Tolerance and withdrawal after 4 weeks of s.c. minipump nicotine infusion

Animals receiving treatment via osmotic minipumps showed a significant effect of nicotine on the time spent in social interaction [$F(2,24)=13.76$, $p<0.0001$], and post-hoc analysis showed that the scores from the animals treated for 4 days ($p<0.01$) and 4 weeks ($p<0.05$) with nicotine were significantly decreased compared with the vehicle controls, see Figure 6.2. However, the animals that had been treated for 4 weeks had significantly higher scores than those that had received nicotine for 4 days ($p<0.01$),

suggesting that again partial tolerance had developed. There were no significant effects of nicotine on locomotor activity [F(2,24)=0.21], see Table 6.1.

The animals that were withdrawn for 24 and 72h from 4 weeks of nicotine administration did not differ from their saline control group in the time they spent in social interaction [F(1,11)=0.7 and F(1,11)=0.1, respectively; see Figure 6.2]. However, the animals tested 24h after withdrawal from nicotine showed a significant increase in locomotor activity compared with saline controls [F(1,11)=5.2, $p<0.05$), but this had disappeared by 72h [F(1,11)=2.0], see Table 6.1.

Plasma Nicotine Levels

There was no significant difference in the plasma concentration of nicotine between rats treated for 4 days or 4 weeks with nicotine in either of the s.c. injection groups (5 and 7 times/week) or in the minipump infusion group, see Table 6.2. Thus, there was no evidence for any development of pharmacokinetic tolerance. Table 6.2 also shows that the plasma nicotine concentration at testing was significantly higher in both the s.c. injection groups than in the minipump group (U=0, $p<0.05$ in both cases). This confirms the higher peak concentrations produced by intermittent injections.

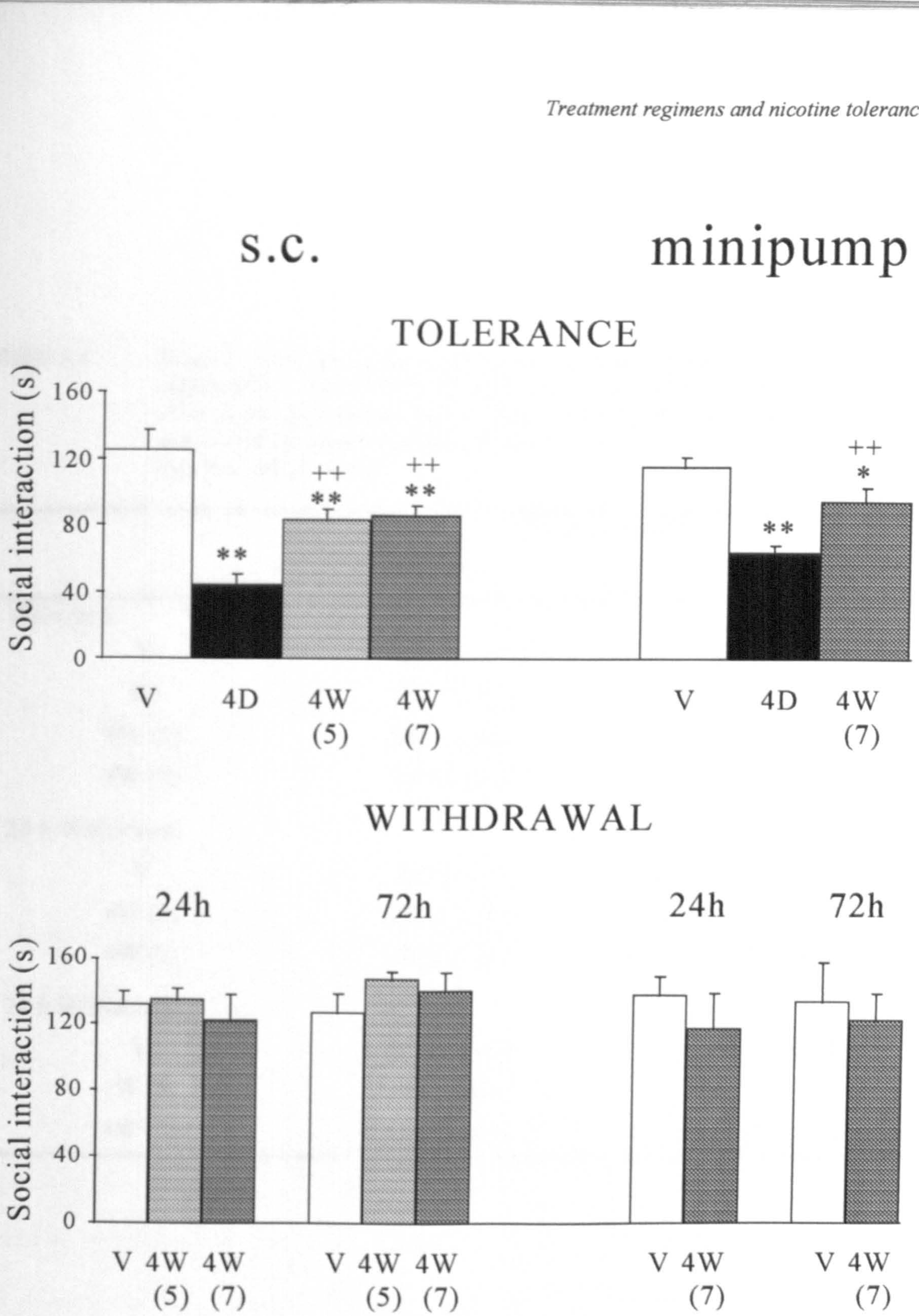

Figure 6.2 Mean (± sem) time (s) spent in social interaction made by rats after vehicle (V) or 4 days (4D) and 4 weeks (4W, 5 or 7 injections/week) of nicotine (0.45 mg/kg/day) administration, either by s.c. injection or minipump infusion (top panel), and 24 and 72h after 4 weeks of nicotine treatment (bottom panel). * $p<0.05$ and $p<0.01$, compared with the vehicle control, and $^{++}$ $p<0.01$, compared with the 4 day group.

Table 6.1 Mean (± sem) locomotor activity (no of beam breaks) made by rats after vehicle (V), 4 days (4D) or 4 weeks (4W, 5 or 7 injections/week) of nicotine (0.45 mg/kg/day) administration, either by s.c. injection or minipump infusion, and 24 and 72h after withdrawal from 4 weeks of nicotine. * $p<0.05$ compared with the vehicle control.

	Locomotor Activity	
	s.c.	Minipump
Tolerance		
V	491.4 ± 19.1	455.1 ± 19.0
4D	412.3 ± 30.7	455.7 ± 31.0
4W (5)	468.1 ± 24.1	-
4W (7)	490.6 ± 25.5	476.7 ± 23.8
24 h Withdrawal		
V	454 ± 30.2	411.1 ± 36.0
4W (5)	425.8 ± 27.0	-
4W (7)	490.2 ± 28.0	516.5 ± 26.4 *
72 h Withdrawal		
V	423.6 ± 24.4	395.9 ± 33.7
4W (5)	509.7 ± 33.6	-
4W (7)	444.5 ± 60.3	455.7 ± 23.2

Table 6.2 Median plasma nicotine levels following 4 days (4D) or 4 weeks (4W, 5 or 7 injections/week) of nicotine administered by either, s.c. injections or constant infusion. ** $p<0.01$ compared to the same treatment in the animals that received constant infusion of nicotine.

Treatment Group	Plasma Nicotine Levels (ng/ml)	n
s.c. injections		
4D	123.1 **	4
4W (5)	105.9	3
4W (7)	134.3 **	4
s.c infusions		
4D	5.7	3
4W (7)	9.6	3

6.4 Discussion

The results of this study have clearly shown that after four days of nicotine treatment (0.45 mg/kg/day) there were decreases in social interaction, without changes in locomotor activity, suggesting specific anxiogenic effects. These effects were very similar, regardless of whether nicotine was given by intravenous injection, subcutaneous injection or infused by minipump. The plasma nicotine concentrations seen in this study after subcutaneous injections are similar to those reported by Shoaib & Stolerman (1999) after intravenous self-administration of nicotine, but these levels were very different from the plasma concentrations in the minipump group. The plasma concentrations in our minipump group are similar to those found by Rowell & Li (1997) following minipump infusions of 0.6 mg/kg/day. These results therefore suggest that, at least after 4 days of treatment, the anxiogenic effects were not related to

the plasma nicotine concentration and perhaps it is simply necessary to reach a certain threshold concentration to see an anxiogenic effect. After an acute dose of nicotine and after 10 days of treatment brain concentrations of nicotine are threefold higher than in plasma (Rowell and Li, 1997; Mansner and Mattila, 1975). It is possible that this difference is further enhanced after 4 weeks of treatment and perhaps to a greater extent in the continuous infusion group, although this was not the case after 10 days of treatment (Rowell and Li, 1997).

The results of the present study strongly suggest that it takes more than 4 weeks for complete tolerance to develop to the anxiogenic effect of this relatively high dose of nicotine, but that the route and manner of nicotine administration is relatively unimportant to the rate of tolerance development. This is in contrast to the rapid rate of development of tolerance to the low dose of nicotine (0.1 mg/kg). The anxiogenic effects of high doses are mediated by stimulation of postsynaptic 5-HT_{1A} receptors in the dorsal hippocampus (Kenny et al, 2000b) and the lateral septal nucleus (Cheeta et al., 2000b). It is therefore possible that different mechanisms and/or rates of tolerance operate in the different brain regions. The brain region mediating the anxiogenic effect of 0.1 mg/kg, that is observed 5 min after injection, is unknown at present.

The results showing persistent anxiogenic effects following 4 weeks of nicotine treatment (0.45 mg/kg/day) support our previous findings that animals that had been self-administering nicotine (0.45 mg/kg/day) for 4 weeks had an anxiogenic response in the social interaction test when they were tested 5 min after their usual self-

administration session. This is important because it shows that a dose of nicotine can cause anxiety and be rewarding. Corrigall and Coen (1989) had suggested that higher doses of nicotine are not self-administered because of their aversive properties. Whilst this is likely in the proconvulsant range and possibly with an extreme level of anxiety, our results raise the possibility that a milder anxiogenic effect is actually rewarding. A very similar argument for the rewarding effects of cocaine has been proposed by Goeders (2001), who has shown that the release of the stress hormones CRH and corticosterone are necessary for cocaine self-administration. The dose of amphetamine that is self-administered is also one that has anxiogenic effects (Lin et al., 1999; Carroll and Lac, 1997; File and Hyde, 1979).

After four weeks of treatment, the anxiogenic effects persisted, but there was development of partial tolerance. This is unlikely to be due to the development of pharmacokinetic tolerance, since there was no significant reduction in nicotine concentrations after four weeks of treatment. Several pharmacodynamic mechanisms are possible. An oppositional mechanism of tolerance is one that involves the progressive recruitment of processes that oppose the acute effect of the drug. Thus, following withdrawal of the drug these processes work unopposed and a behavioural response is seen in the opposite direction of the acute drug effect (Young & Goudie, 1995). There was no evidence for an oppositional mechanism, since no anxiolytic withdrawal responses were observed at either 24 or 72h after the end of nicotine treatment. However, it is possible that the absence of a withdrawal response was because tolerance had not fully developed. An alternative mechanism of tolerance is a

decremental one, in which the behavioural impact of a drug is reduced, but which is without behavioural consequence in the absence of the drug (Young & Goudie, 1995). The pattern of results in this study would fit with a decremental process, such as receptor desensitisation, which has been observed in vitro with very low nicotine concentrations (Bencherif et al., 1995; Grady et al., 1994; Rowell and Hillebrand, 1994; Marks et al., 1993a, b). However, in an in vivo study receptor desensitisation was only found with plasma nicotine concentrations of 24-87 ng/ml, and not with a concentration of 9 ng/ml (Benwell et al., 1995). Since our minipump group had a concentration of 9.6 ng/ml our results suggest that receptor desensitisation might occur at this concentration in at least some brain regions.

There are also clear differences in the responses that can be seen on withdrawal from chronic treatment with the low and high doses of nicotine. Following 7 days of treatment with the low dose, an anxiogenic effect was seen 72h after the last dose (Chapter 2). Following the 4 weeks of treatment with the high dose, there were no changes in social interaction at either 24 or 72h in the present study or when rats were self-administering nicotine (Chapter 5). It had been speculated that the lack of a withdrawal response in the rats that had been self-administering nicotine was due to their having had previous experience of 72h withdrawal periods each weekend. However, this does not seem to be the crucial factor since there was no change in social interaction following withdrawal in the rats with daily injections or with constant infusions of nicotine. The results suggest that kindling of an anxiogenic response does not occur after repeated nicotine withdrawals. This has also been found following

repeated benzodiazepine withdrawals, although kindling of convulsions does occur (Ward and Stephens, 1998). Furthermore, whilst repeated administration of the benzodiazepine partial inverse agonist FG 7142 kindles seizures, it does not kindle anxiety (Taylor et al., 1988). The separation of anxiety and seizures was discussed by Pellow (1985). The only changes that we found when testing during withdrawal from the 4 weeks of nicotine treatment was that in two of the groups (i.v. injections 5 times/week and continuous minipump infusion) there was a significant increase in locomotor activity. However, it would not seem correct to interpret the increased locomotor activity as a true withdrawal response, since locomotor activity was not decreased by either acute or 4 days of nicotine treatment. Furthermore, sensitisation to a locomotor stimulant effect, as measured in photocell activity cages, has been found (Zubaran et al., 2000) after 3 weeks of nicotine treatment (0.4 mg/kg/day). Whilst this was not detected in the conditions of the social interaction test, there was a trend towards increased locomotor activity in the 4 week intravenous nicotine group. The lack of locomotor sensitisation could be because two animals are present in the social interaction test and this could modify nicotine's effects on locomotor activity, as has been found for chlorpromazine (File and Pope, 1974b). Locomotor depressant effects have been found in the social interaction test following an injection of 0.5 mg/kg nicotine, but this was in the hooded Lister strain of rat. It is possible that there is a strain difference in sensitivity to these effects.

In conclusion, the results of the present study show, somewhat surprisingly, that the treatment regimen did not affect the rate of development of tolerance to the anxiogenic

effects of a high dose of nicotine, despite strikingly different levels of peak plasma concentration, and despite differences in the patterning of nicotine treatment.

CHAPTER 7

The dorsal raphé nucleus is a crucial structure mediating nicotine's anxiolytic effects and is involved in the development of tolerance and withdrawal responses

7.1 Introduction

The dorsal hippocampus and the lateral septum have been identified as important neuroanatomical substrates mediating nicotine's anxiogenic effects (Ouagazzal et al, 1999b; File et al, 1998). Furthermore, the anxiogenic effects of nicotine were antagonised by co-administration of the specific 5-HT_{1A} receptor antagonist WAY 100,635 (Cheeta et al, 2000b; Kenny et al, 2000b), suggesting an important role for 5-HT_{1A} receptors in these brain areas in mediating nicotine's anxiogenic effects. An interaction between nicotinic and 5-HT_{1A} receptors have been implicated in a number of nicotine's other behavioural effects (Rasmussen et al, 1997; Damaj et al, 1994; Riekkinen et al, 1994), and may therefore also exist for nicotine's anxiolytic actions.

The 5-HT projections to the lateral septum arise from the DRN (Vertes, 1991; Andersen et al, 1983), and earlier research has demonstrated that direct administration of the specific 5-HT_{1A} receptor agonist 8-OH-DPAT into the DRN induces an anxiolytic effect in the social interaction test (File et al, 1996a; Hogg et al, 1994; Higgins et al, 1988). This suggested that the DRN might be a good substrate for mediating the anxiolytic effects of nicotine. Therefore, this study investigated the

effects of direct administration of nicotine into the DRN in the social interaction test. To investigate whether there is a link between somatodendritic 5-HT_{1A} receptors and nicotine's anxiolytic actions, the ability of WAY 100,635 to reverse the anxiolytic action of intra-DRN nicotine was investigated.

In the social interaction test, tolerance to both the acute anxiogenic and anxiolytic effects of nicotine have been reported (Chapter 2). To date a neuroanatomical site mediating tolerance to the anxiolytic effects of nicotine has not yet been reported, and therefore this study explored the role of the DRN.

In the social interaction test, an anxiogenic response is seen 72h following the termination of 7 days of nicotine administration (Chapter 2). The DRN has been implicated in the increased anxiety seen during nicotine withdrawal. Administration of ondansetron into the DRN antagonised the anxiogenic withdrawal response measured in the black/white crossing test (Costall et al, 1990c). Rasmussen & Czachura (1997) have also shown that on withdrawal from chronic nicotine, sensitivity to the 5-HT_{1A} agonist, 8-OH-DPAT in the DRN is increased. Thus, this study also investigated the role of the DRN in mediating the increased anxiety detected in the social interaction test on withdrawal from chronic nicotine.

7.2 Materials and Methods

Animals

Male hooded Lister rats (Charles River, Margate, Kent, UK) weighing between 220-250g were used in all experiments. For Experiment 1, rats were housed singly following surgery and allowed to recover for 7 days prior to behavioural testing. For Experiment 2, following surgery rats were singly housed, and allowed to recover for 4 days prior to the start of chronic injections. The unoperated rats used in each experiment were housed singly for the same length of time as their operated test partners. Food and water were freely available for all animals. The room in which animals were housed was lit with dim light and maintained at 22°C. Lights were on from 0700-1900h.

Apparatus

The social interaction test, see Chapter 2 for description.

Surgery

Surgery was conducted as described an in Chapter 3 with the exception that in Experiment 1a, for unilateral cannulation of the DRN, 12 mm long steel guide cannulae (23 gauge) were positioned at 7.4mm posterior to bregma, ± 2.2 lateral and vertical -4.7 mm at an angle of 19°, thus siting them 2 mm above the target area (according to the atlas of Paxinos & Watson, 1986). For Experiments 1b and 2, the dorsal ventral co-

ordinate was changed to -4.5mm, but all other co-ordinates were those used in Experiment 1a. In all experiments, cannulae were kept patent using 12 mm long stainless steel stylets (30 gauge).

Drugs and chemicals

For the chronic sub-cutaneous injections, (-)-nicotine hydrogen tartrate (Sigma, Poole, UK) was dissolved in distilled water, in a volume of 1 ml/kg body weight and a dose of 0.1 mg/kg was used; control animals received equal volume injections of distilled water. For the central injections, (-)-nicotine hydrogen tartrate and WAY 100,635 (Sigma, Poole, UK) were dissolved in aCSF (composition as stated in Chapter 3). In order to perform antagonism studies, agonist and antagonist compounds were co-administered together in a single injection. All injection volumes were 0.5 µl. and injections were made over a period of 30 s using a CMA/102 microdialysis pump and the needles were left in position for a further 30 s to allow drug diffusion; control animals received 0.5 µl infusions of aCSF. All doses are given as free base.

Behavioural Testing

Social Interaction Test

In order to familiarise rats to the social interaction test arena, each rat was placed individually in the test arena, under high light conditions of illuminance (300 lux) for a 5 min familiarisation trial on each of the 2 days prior to social interaction testing. In all experiments, rats were allocated to pairs, such that members of a pair did not differ in

weight by more than 10 g. In experiments in which behaviour was assessed after a s.c. injection, the treated rat was injected with vehicle or nicotine and tested 30 min later, with an untreated rat, and behaviour initiated by the treated rat was scored. Within each experiment involving rats with cannulation of the DRN, three minutes after central injection, the operated rat was placed together with its unoperated partner in the test arena and social interaction initiated by the treated rat was scored. In all experiments, social interaction was scored for 4.5 min by an observer blind to the drug treatment. Rats were tested between 0830-1300h in an order randomised for drug treatment, and the test arena was cleaned with a paper towel after each trial.

Allocation to drug groups

In each experiment, the rats were randomly allocated to the treatment groups as specified below. The initial dose-response data following intra-DRN nicotine were collected from separate experimental test days. Since there were no differences in the baseline scores of social interaction in the aCSF treated animals between the different days, these control data were pooled. The numbers in parentheses indicate the numbers of rats in each group with verified cannulae placements.

Experiment 1

(a) Effects of intra-DRN injections of nicotine

aCSF (n=16); nicotine: 5ng (n=9); 10ng (n=4); 100ng (n=5); 1 μg (n=5): 4 μg (n=5).

(b) Reversal of the anxiolytic effect of nicotine by WAY 100,635

aCSF (n=11); nicotine 5ng (n=10); nicotine 5ng + WAY 100,635 200ng (n=9); Way 100635 200ng (n=9)

Experiment 2

(a) Effects of DRN injections of nicotine in animals chronically treated with sub-cutaneous nicotine

Animals were randomly allocated to treatment with either vehicle or nicotine (0.1 mg/kg, s.c.) for 6 days. On the 7th day, no s.c. injections were given, and the animals were challenged with an intra-DRN injection. Animals chronically treated with vehicle were randomly allocated to the following DRN injections: aCSF (n=11), nicotine 2.5ng (n=8), 5ng (n=10), or 7.5ng (n=7). Animals that were treated with nicotine 0.1mg/kg s.c. daily were also tested on the 7th day following a DRN injection of either aCSF (n=9), nicotine 2.5ng (n=4), 5ng (n=7), or 7.5ng (n=8).

(b) Reversal of the anxiogenic response observed following withdrawal from chronic nicotine

Animals were randomly allocated to treatment with either vehicle or nicotine (0.1mg/kg, s.c.) for 7 days. Animals that were chronically treated with vehicle (n=9), received an injection of aCSF into the DRN 72h after their last s.c. injection. Animals that had received chronic nicotine were split into two groups and received an intra-DRN injection of aCSF (n=13), or nicotine 5ng (n=6) 72h following the termination of chronic treatment. A final group of animals that had also received 7 days of 0.1mg/kg

nicotine were tested following a s.c. injection of nicotine 0.1mg/kg s.c. (n=6) following 72h withdrawal from chronic nicotine.

Histology

At the end of behavioural testing all animals were sacrificed, the brains removed and the injection sites verified histologically (according to the atlas of Paxinos & Watson, 1986) by a person blind to drug treatment. Cannulae placements that were located within the DRN were found to be within the anterior planes of -7.30 to -7.80mm to bregma. Figure 7.1 depicting coronal slices through the DRN shows the target sites as shaded, and the tips of the injection needles for animals that were excluded (not in the target area) by the filled squares. Subjects in which the injection site was located outside the DRN were discarded from statistical analysis.

Statistics

Data were analysed by one or two-way ANOVA, as appropriate. A comparison between individual groups was then made with Fisher's least squared difference (LSD) test. When there were significant differences in both measures of anxiety and motor activity, ANCOVA were conducted in order to determine the independence of the changes. The data and significance levels shown in the tables and figures are those that remain after ANCOVA.

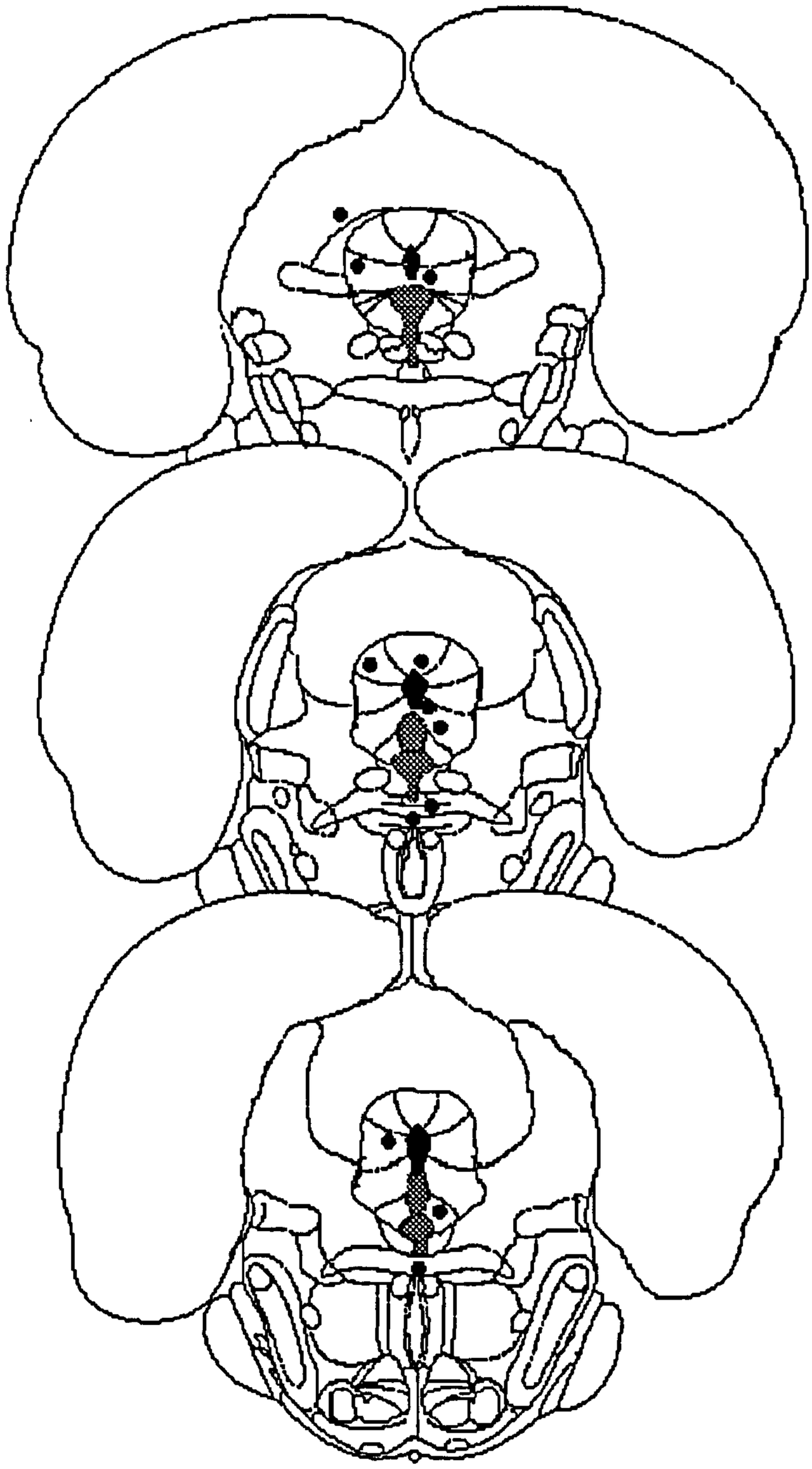

Figure 7.1 Coronal sections of rat brain showing the target areas (shaded) of the dorsal raphé nucleus. Placements falling outside the target area are shown by filled squares marking the tip of the injection needle. Values give the distance in mm anterior to posterior to bregma, according to the atlas of Paxinos & Watson (1986).

7.3 Results

Experiment 1

(a) Effects of intra-DRN injections of nicotine

Infusion of nicotine into the DRN showed dose dependent effects on the time spent in social interaction, [$F(5,38)=17.04$, $p<0.0001$], and post hoc tests showed that nicotine (5 and 10ng) significantly increased social interaction, see Figure 7.2. At these same doses nicotine had no significant effects on locomotor activity (see Table 7.1), and thus seemed to be having a specific anxiolytic effect. However, at the higher dose of 4 μg, nicotine significantly decreased social interaction (see Figure 7.2), but this was also accompanied by a significant decrease in locomotor activity, see Table 7.1. In cases such as this, an analysis of covariance allows one to determine whether these are two independent effects or whether the other causes the decrease in one. Nicotine (4μg) still significantly decreased social interaction [$F(1,18)=12.9$, $p<0.005$] even when hypoactivity was accounted for, and thus seemed to be having a specific anxiogenic effect. Nicotine no longer had any effect on locomotor activity [$F(1,18)=1.5$] when the decrease in social interaction was accounted for, see Table 7.1. Thus, in this experiment the decrease in locomotor activity after nicotine administration was secondary to the increase in anxiety elicited by nicotine (4μg).

(b) *Reversal of the anxiolytic effect of nicotine by WAY 100,635*

Once again nicotine (5ng) significantly increased the time spent in social interaction [$F(1,35)=10.13$, $p<0.005$; Figure 7.3], and this effect was reversed by the specific 5-

HT_{1A} antagonist WAY 100,635, which had no effect when administered alone [nicotine x WAY 100,635 interaction, $F(1,35)=4.83$, $p<0.05$; Figure 7.3]. Neither nicotine nor WAY 100,635 changed locomotor activity [$F(1,35)<1.0$ in both cases; Table 7.1].

Table 7.1 Mean (± sem) locomotor activity (beam breaks) of rats in Experiments 1a and 1b, in the high light, familiar condition in the social interaction test following an injection into the dorsal raphé nucleus of (Experiment 1a) artificial cerebrospinal fluid (aCSF) or nicotine (5-4000ng), or (Experiment 1b) nicotine (5ng) plus WAY 100,635 (200ng), or WAY 100,635 (200ng). n= number of rats in each group with verified cannulae placements. +difference compared with the appropriate aCSF control group that did not remain significant after ANCOVA.

Drug Treatment	Locomotor Activity	n
aCSF	307.06 ± 20.8	16
Nicotine 5ng	345.11 ± 30.0	9
Nicotine 10ng	277.50 ± 58.8	4
Nicotine 100ng	306.60 ± 48.5	5
Nicotine 1000ng (1µg)	383.40 ± 24.6	5
Nicotine 4000ng (4 µg)	208.95 ± 20.8*	5
aCSF	333.54 ± 23.5	11
Nicotine 5ng	328.00 ± 21.43	10
Nicotine 5ng and WAY 200ng	315.11 ± 27.93	9
WAY 200ng	355.66 ± 29.5	9

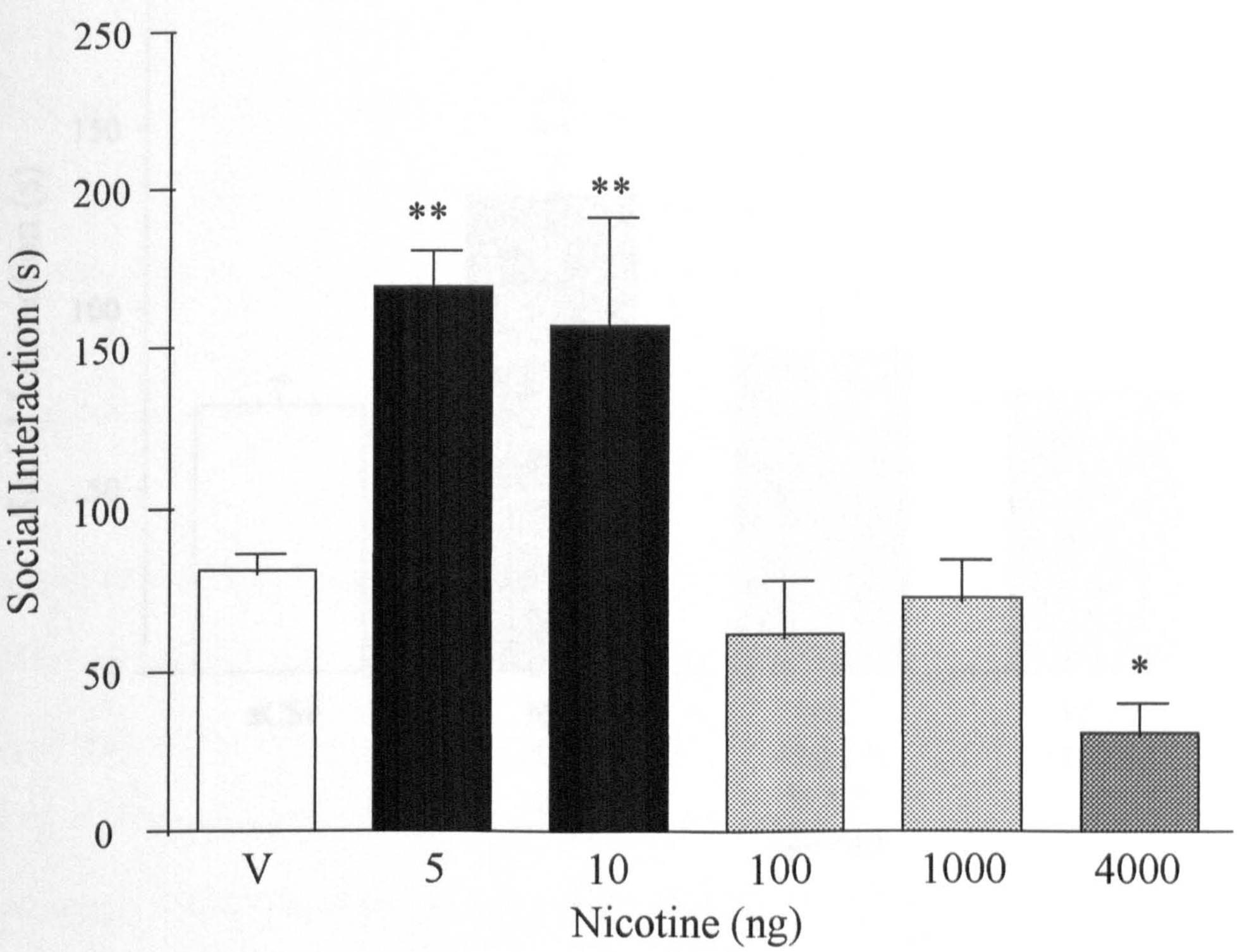

Figure 7.2 Mean (± sem) time spent in social interaction by rats in the high light, familiar test condition following an injection into the dorsal raphé nucleus of artificial cerebrospinal fluid (aCSF) or nicotine (5-4000ng). *p<0.05 and **p<0.01 compared with the aCSF control group.

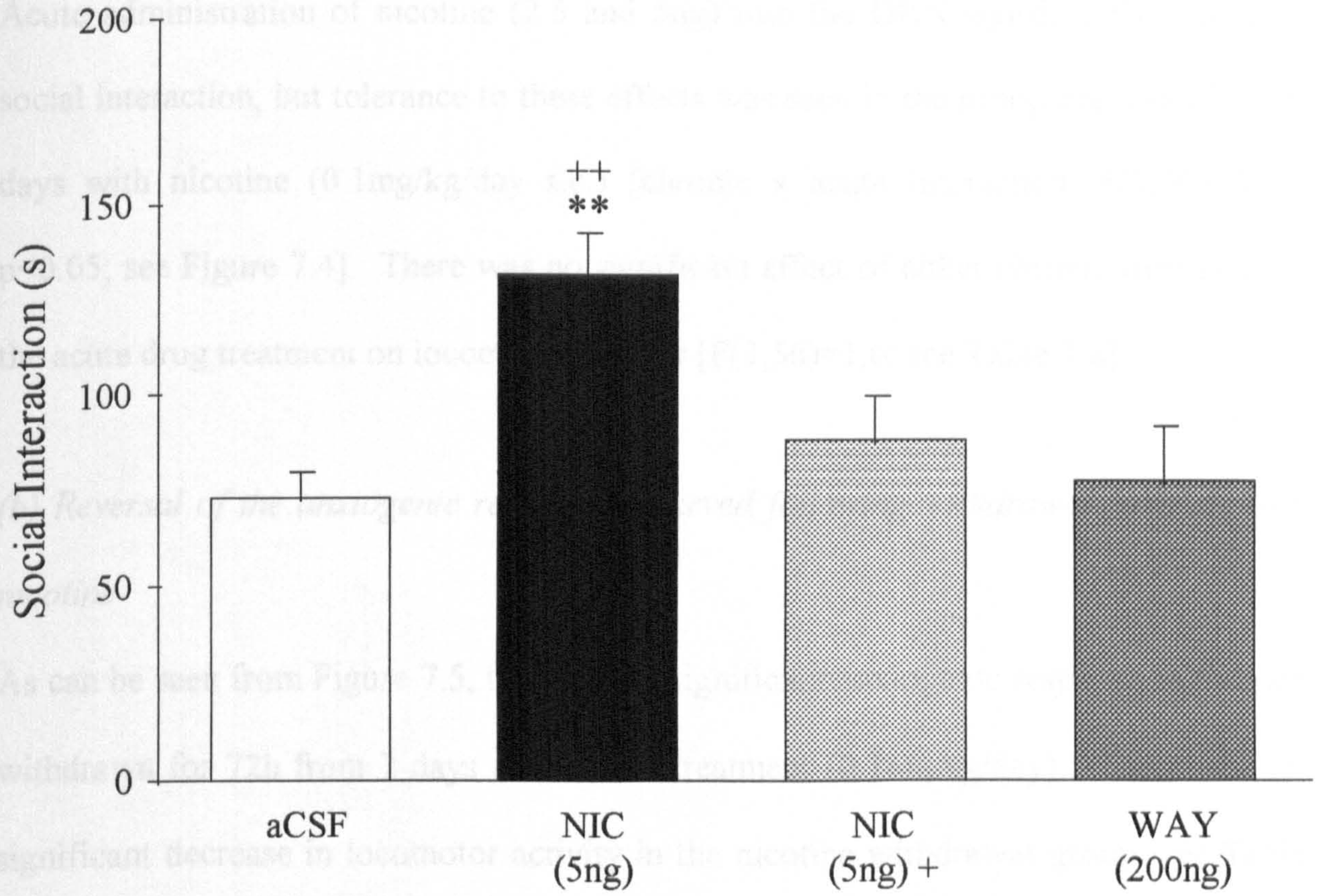

Figure 7.3 Mean (± sem) time spent in social interaction by rats in the high light, familiar test condition following an injection into the dorsal raphé nucleus of artificial cerebrospinal fluid (aCSF), nicotine (5ng) (NIC 5ng), nicotine (5ng) & WAY 100,635 (200ng) (NIC 5ng + WAY 200ng), and WAY 100635 (200ng) (WAY 200ng). **p<0.01 compared with aCSF control group, ++p<0.01 compared with NIC + WAY group.

Experiment 2

(a) Effect of DRN injections of nicotine in animals chronically treated with sub-cutaneous nicotine

Acute administration of nicotine (2.5 and 5ng) into the DRN significantly increased social interaction, but tolerance to these effects was seen in the group pre-treated for 6 days with nicotine (0.1mg/kg/day s.c.) [chronic x acute interaction: $F(3,56)=3.34$, $p<0.05$; see Figure 7.4]. There was no significant effect of either chronic treatment or the acute drug treatment on locomotor activity [$F(3,56)=1.6$; see Table 7.2].

(b) Reversal of the anxiogenic response observed following withdrawal from chronic nicotine

As can be seen from Figure 7.5, there was a significant anxiogenic response in the rats withdrawn for 72h from 7 days of nicotine treatment (0.1mg/kg/day). There was no significant decrease in locomotor activity in the nicotine withdrawal group (see Table 7.3), therefore there seems to be a specific anxiogenic effect in these animals. The anxiogenic withdrawal response was reversed with nicotine either s.c. or into the DRN [$F(3,30)=8.07$, $p<0.0001$]. In the latter case, this was accompanied by an increase in locomotor activity (Table 7.3).

Placement Errors

The scores from animals with placements falling outside the DRN but receiving doses of 5-10ng nicotine were low ($x = 49.0$; range 7-127s). In comparison, the same doses in the DRN produced much higher scores ($x = 163$; range 98-260s). These values

provide evidence for the anatomical specificity of the results following DRN injections.

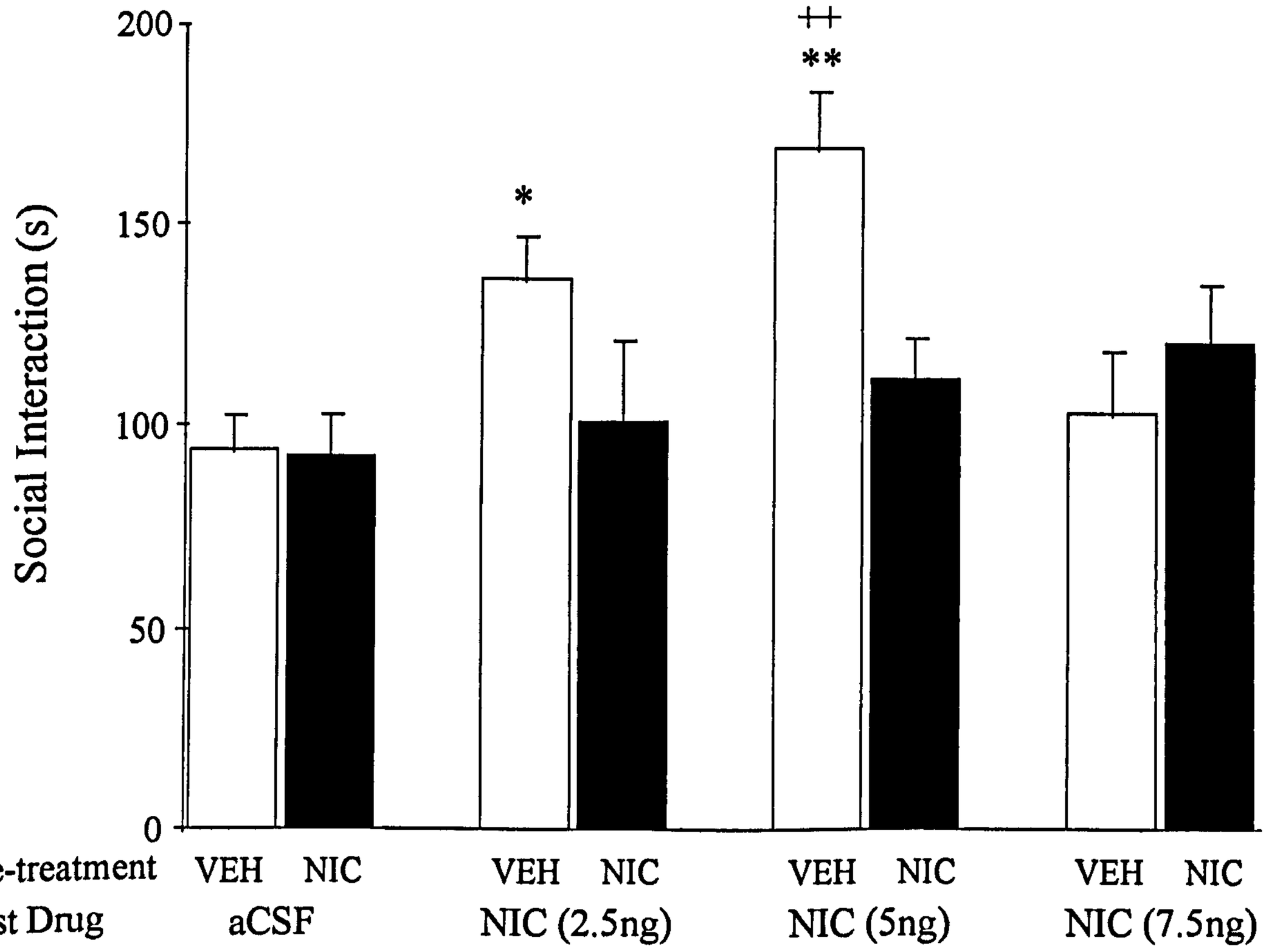

Figure 7.4 Mean (± sem) time spent in social interaction by rats in the high light, familiar test condition that had been treated for 6 days (chronic treatment) with vehicle (VEH) or nicotine (0.1 mg/kg, s.c.; NIC) and then tested (test drug) following an injection into the dorsal raphé nucleus with artificial cerebrospinal fluid (aCSF) or nicotine (2.5-7.5ng). *$p<0.05$, **$p<0.01$ compared with rats treated with vehicle for 6 days and then tested with aCSF in the dorsal raphé nucleus. $^{++}p<0.01$ compared with rats treated with nicotine for 6 days and then tested following administration of 5ng of nicotine into the dorsal raphé nucleus.

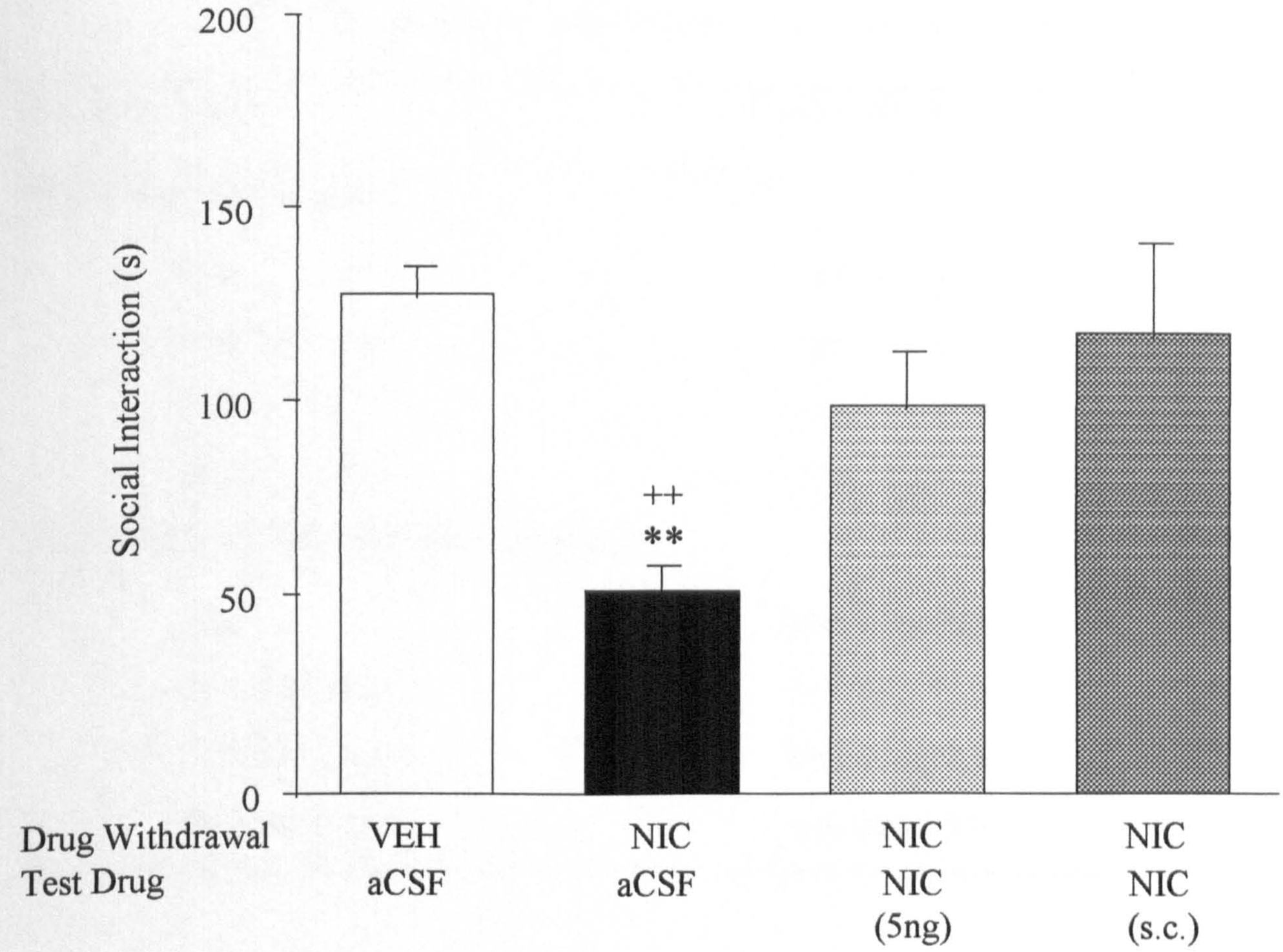

Figure 7.5 Mean (± sem) time spent in social interaction by rats that were in 72h withdrawal following treatment for 7 days (drug withdrawal) with vehicle (VEH) or nicotine (0.1 mg/kg, s.c.; NIC) and then challenged (test drug) with an injection into the dorsal raphé nucleus of artificial cerebrospinal fluid (aCSF), nicotine (5ng) (NIC 5ng), or a s.c. injection of nicotine (0.1mg/kg) (NIC s.c.). Rats were tested in the high light, familiar test condition. **$p<0.01$ compared with animal treated for 7 days with vehicle and tested following 72h withdrawal with aCSF; $^{++}p<0.01$ compared with rats treated with nicotine for 7 days and then tested following 72h withdrawal with administration of either 5ng of nicotine into the dorsal raphé nucleus or a s.c. injection of 0.1mg/kg nicotine.

Table 7.2 Mean (± sem) locomotor activity (beam breaks) of rats that had been treated for 6 days with vehicle or nicotine (0.1 mg/kg/day, s.c.) and then challenged with an injection into the dorsal raphé nucleus of artificial cerebrospinal fluid (aCSF) or nicotine (2.5-7.5ng). Rats were tested in the high light familiar test condition. n= number of rats in each group with verified cannulae placements.

Drug Treatment	Locomotor Activity	n
Pre-treatment: Vehicle		
aCSF	333.54 ± 23.5	11
Nicotine 2.5ng	329.62 ± 16.2	8
Nicotine 5ng	328.71 ± 21.4	10
Nicotine 7.5ng	349.71 ± 4.5	7
Pre-treatment: Nicotine (0.1 mg/kg s.c)		
aCSF	390. 55 ± 25.0	9
Nicotine 2.5ng	311.50 ± 31.3	4
Nicotine 7.5ng	383.00 ± 18.5	7
Nicotine 7.5ng	349.62 ± 35.6	8

Table 7.3 Mean (± sem) locomotor activity (beam breaks) of rats withdrawn for 72h following treatment for 7 days with vehicle or nicotine (0.1 mg/kg/day, s.c.). On the test day animals received an injection into the dorsal raphé nucleus of artificial cerebrospinal fluid (aCSF) or nicotine (5ng) or nicotine (0.1mg/kg, s.c.). n = number of rats in each group with verified cannula placement where applicable. *p<0.05 compared with the chronic nicotine group tested following intra-DRN aCSF.

Chronic treatment	Treatment at test	Locomotor Activity	n
Vehicle	aCSF	282 ± 31.0	9
Nicotine	aCSF	240 ± 14.5	13
Nicotine	Nicotine 5ng	343 ± 42.9*	6
Nicotine	Nicotine 0.1mg/kg s.c.	261 ± 25.1	6

7.4 Discussion

The present study demonstrated that in the social interaction test, administration of low doses of nicotine into the DRN induced anxiolytic effects, which were not accompanied by changes in locomotor activity, suggesting that nicotine was acting specifically to reduce anxiety. This effect was completely reversed by co-administration of a behaviourally inactive dose of the 5-HT_{1A} receptor antagonist, WAY 100,635. Previously it has been demonstrated that the anxiogenic effects of nicotine seen in the social interaction test and elevated plus maze following intra-hippocampal or intra-septal administration are also reversed by co-administration of WAY 100,635 (Cheeta et al, 2000a; Kenny et al, 2000b). Therefore, these findings suggest that the link between the 5-HT_{1A} receptors and nicotinic receptors in mediating the anxiogenic actions of nicotine, also extends to the DRN mediated anxiolytic action of nicotine. This finding adds to growing evidence that there may be a rather general 5-HT_{1A}-nicotinic receptor interaction in mediating certain behavioural effects of nicotine (Rasmussen et al, 1997; Damaj et al, 1994; Riekkinen et al, 1994; Hilleman et al, 1994, 1992; West et al, 1991).

Previously it has been demonstrated that DRN administration of the 5-HT_{1A} receptor agonist, 8-OH-DPAT in the social interaction test induced an anxiolytic effect similar to that seen with nicotine (Hogg et al, 1994; Higgins et al, 1988). The mechanism that has been assumed to mediate the anxiolytic effects of 8-OH-DPAT is via stimulation of the 5-HT autoreceptors in the raphé nucleus leading to a reduction in 5-HT neuronal firing (Sprouse & Aghajanian, 1987), and a subsequent decrease in 5-HT release in

terminal regions of the limbic system. Nicotine stimulates the release of 5-HT in the DRN (Mihailescu et al, 1998), and the reversal of its anxiolytic effect by WAY 100,635 suggest that this action is mediated by indirectly stimulating the somatodendritic 5-HT_{1A} receptors. A recent study provides physiological evidence in support of this proposition, since it was demonstrated that i.v. nicotine administration reduced dorsal raphé firing in anaesthetised rats, an effect which could also be antagonised by WAY 100,635 (Engberg et al, 2000). However, they found no effect of nicotine on 5-HT firing when it was microiontophorised into the DRN. Nevertheless, with this technique, only very small amounts of nicotine are released, and this is unlikely to activate the receptors on the afferent 5-HT terminals on which nicotine induced 5-HT release occurs. The anxiogenic effect of the high dose of nicotine (4μg) into the DRN may result from an action at heteroreceptors leading to the release of other neurotransmitters such as noradrenaline. Alternatively, it could be the result of diffusion of nicotine outside the DRN and into surrounding structures. This diffusion could explain the lack of effect seen with intermediate doses of nicotine, since an anxiogenic effect mediated by these areas adjacent to the DRN could counteract the anxiolytic effect mediated within the DRN. The low scores from animals where the cannula placements fell outside the DRN would strengthen this possibility. A similar result was seen with the 5-HT_{1A} agonist 8-OH-DPAT. This had an anxiolytic effect when administered in the DRN, but a marked anxiogenic effect when the placements fell outside (Gonzalez & File, 1997; File & Gonzalez, 1996).

In the present study, we have identified the DRN as a crucial neuroanatomical substrate involved in mediating the development of tolerance to nicotine's anxiolytic actions. Rapid tolerance was shown by a lack of response to intra-DRN nicotine (2.5 and 5ng) in chronically treated animals. Chronic nicotine treatment also did not change the response to a challenge with 7.5ng nicotine. However, Stolerman et al (1974) reported that one single previous systemic administration of nicotine (0.75 mg/kg i.p.) could produce a shift to the right of the dose response curve for a second dose by a factor of about 2.4, which is similar to our results. In the study investigating tolerance to nicotine's anxiolytic actions, acutely administered 7.5ng nicotine was without effect, although an anxiolytic effect was reported following 10ng nicotine in the initial dose response study. Therefore, there does seem to be a small difference in sensitivity to nicotine in these two experiments, although social interaction scores in the aCSF treated controls groups did not differ between these two experiments. However, an anxiolytic effect following 5ng nicotine was consistently observed in all experiments suggesting this to be an optimum dose within an approximate dose range.

The anxiogenic response seen 72h after the end of chronic treatment seems to be a withdrawal response, because it could be reversed by either sub-cutaneous or intra-DRN nicotine. The existence of a withdrawal response is evidence of an oppositional mechanism of tolerance. A down-regulation or desensitisation of nicotinic or 5-HT_{1A} receptors could account for the development of tolerance, but would not result in anxiogenic response when the drug was withdrawn. Alternatively, tolerance could be mediated by a compensatory change such as increased firing of the raphé neurones,

which would lead to increased release when the nicotine was withdrawn. This increased release in projection areas, such as the lateral septum, would lead to an anxiogenic response (Cheeta et al, 2000b). A similar mechanism in the median raphé nucleus has been shown to mediate the anxiogenic response that occurs on withdrawal from chronic benzodiazepine treatment (Andrews et al, 1997). In this case, it was shown that during benzodiazepine withdrawal there was enhanced sensitivity to 8-OH-DPAT in the median raphé. Rasmussen & Czachura (1997) have shown that during nicotine withdrawal there is enhanced sensitivity to the inhibitory effects of 8-OH-DPAT on DRN firing, suggesting that withdrawal from nicotine results in increased DRN firing and subsequent 5-HT release.

In summary, the results from the present series of experiments, suggest that the anxiolytic effects of nicotine in the social interaction test are mediated by the 5-HT_{1A} receptors in the DRN. Changes in this structure also mediate the development of tolerance to the effects of nicotine and the anxiogenic response that is detected during nicotine withdrawal. Evidence from other experiments suggests that this withdrawal response might also be mediated by 5-HT_{1A} receptors. Therefore, further studies are needed to investigate the role of the 5-HT_{1A} receptors in the development of tolerance and the expression of nicotine withdrawal responses. If the above hypothesis is correct for the anxiogenic effect seen after termination of nicotine treatment it would be interesting to investigate if the effect could be reversed by administration of WAY 100,635 into the dorsal hippocampus or lateral septum.

CHAPTER 8

The role of the dorsal hippocampus in the development of tolerance to nicotine's anxiogenic effect in the social interaction test

8.1 Introduction

The dorsal hippocampus was found to be one brain region mediating the anxiogenic effect of nicotine in the social interaction test (File et al., 1998). The 5-HT_{1A} receptors were implicated in this action, because co-administration into the dorsal hippocampus of the 5-HT_{1A} receptor antagonist, WAY 100,635, reversed the anxiogenic effect of nicotine (Kenny et al., 2000a). Furthermore, it has been shown that nicotine increases 5-HT release in the dorsal hippocampus (Kenny et al., 2000b) and that glycine significantly enhances this action of nicotine (File et al., 2000b).

In the social interaction test, anxiogenic effects have been found 30 min after systemic administration of high (0.5 & 1 mg/kg) doses of nicotine (File et al., 1998). Interestingly, an anxiogenic effect was also found 5 min after a low (0.1 mg/kg) dose of nicotine (Chapter 2), a dose that has an anxiolytic effect 30 min after systemic injection. After 7 days of nicotine treatment (0.1 mg/kg/day), tolerance developed to the anxiogenic and anxiolytic effects of subcutaneous administration of this dose (Chapter 2), and to the anxiolytic effects of administration of nicotine into the DRN (Chapter 7). The purpose of the present experiment was to explore whether this same

pre-treatment regimen resulted in tolerance to the anxiogenic effect of nicotine administered into the dorsal hippocampus. Animals were therefore treated for 6 days with nicotine (0.1 mg/kg, s.c.) or vehicle and then tested in the social interaction test after a dorsal hippocampal injection with nicotine. The dose selected for hippocampal administration was 1μg, which has been shown in previous studies to be a dose that elicits an anxiogenic effect (Kenny et al., 2000a; File et al., 1998). In order to investigate a possible mechanism underlying the development of tolerance to the anxiogenic effect of nicotine in the dorsal hippocampus, [^{3}H]-5-HT release was measured in superfused dorsal hippocampal slices taken from rats treated for 6 days with nicotine (0.1 mg/kg, s.c.), in order to determine whether tolerance developed to the effects of nicotine on [^{3}H]-5-HT release in this area.

8.2 Materials and Methods

Animals

Male hooded Lister rats (Charles River, Margate, Kent, UK) weighing between 220-250g were housed singly. Rats that had undergone surgery were allowed to recover for 4 days prior to the start of chronic injections. Food and water were freely available, and the room in which they were housed was lit with dim light and maintained at 22°C. Lights were on from 0700-1900h.

Apparatus

The social interaction test, see Chapter 2 for description.

Surgery

Surgery was conducted as described in Chapter 3.

Drugs and chemicals

For the chronic subcutaneous injections, (-)-nicotine hydrogen tartrate (Sigma, Poole, UK) was dissolved in distilled water, in a volume of 1 ml/kg body weight and a dose of 0.1 mg/kg was used; control animals received equal volume injections of distilled water. For the central injections, (-)-nicotine hydrogen tartrate was dissolved in aCSF. Injections were 0.5µl, and were made over a period of 30s using a CMA/102 microdialysis pump (Biotech Instruments Ltd, Stockholm, Sweden) and the needles were left in position a further 30s to allow drug diffusion; control animals received 0.5µl infusions of aCSF. All doses are given as free base.

[^{3}H]-5-HT creatine sulphate was obtained from Amersham International (Amersham, Buckinghamshire, UK). Krebs bicarbonate buffer of the following composition was used (mM): NaCl 118, KCl 4.8, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2 and glucose 9.5. The medium was gassed continuously with 95% O2 / 5% CO_2 and contained (µM) pargyline 50, ascorbic acid 100 and EDTA 35.

Behavioural Testing

Effect of a dorsal hippocampal injection of nicotine after chronic nicotine treatment

Animals were randomly allocated to treatment with either vehicle or nicotine (0.1 mg/kg, s.c.) for 6 days. Within each of the two chronic treatment groups, animals were randomly allocated to the following hippocampal injection groups: aCSF or (-)nicotine (1μg). The numbers in each group ranged from 7-9 after verification of the cannula placements. On the 7th day, no s.c. injections were given but rats were tested with an unoperated, uninjected partner in the social interaction test 3 min after a bilateral injection into the hippocampus. Only interactions initiated by the operated rats were scored.

Histology

At the end of behavioural testing all animals were sacrificed, the brains removed and the injection sites verified histologically (according to the atlas of Paxinos & Watson, 1986) by a person blind to drug treatment. Figure 8.1, depicting coronal slices through the dorsal hippocampus, shows the target site and the positions of the tips of the injection needles for the rats excluded from statistical analysis because of placement errors.

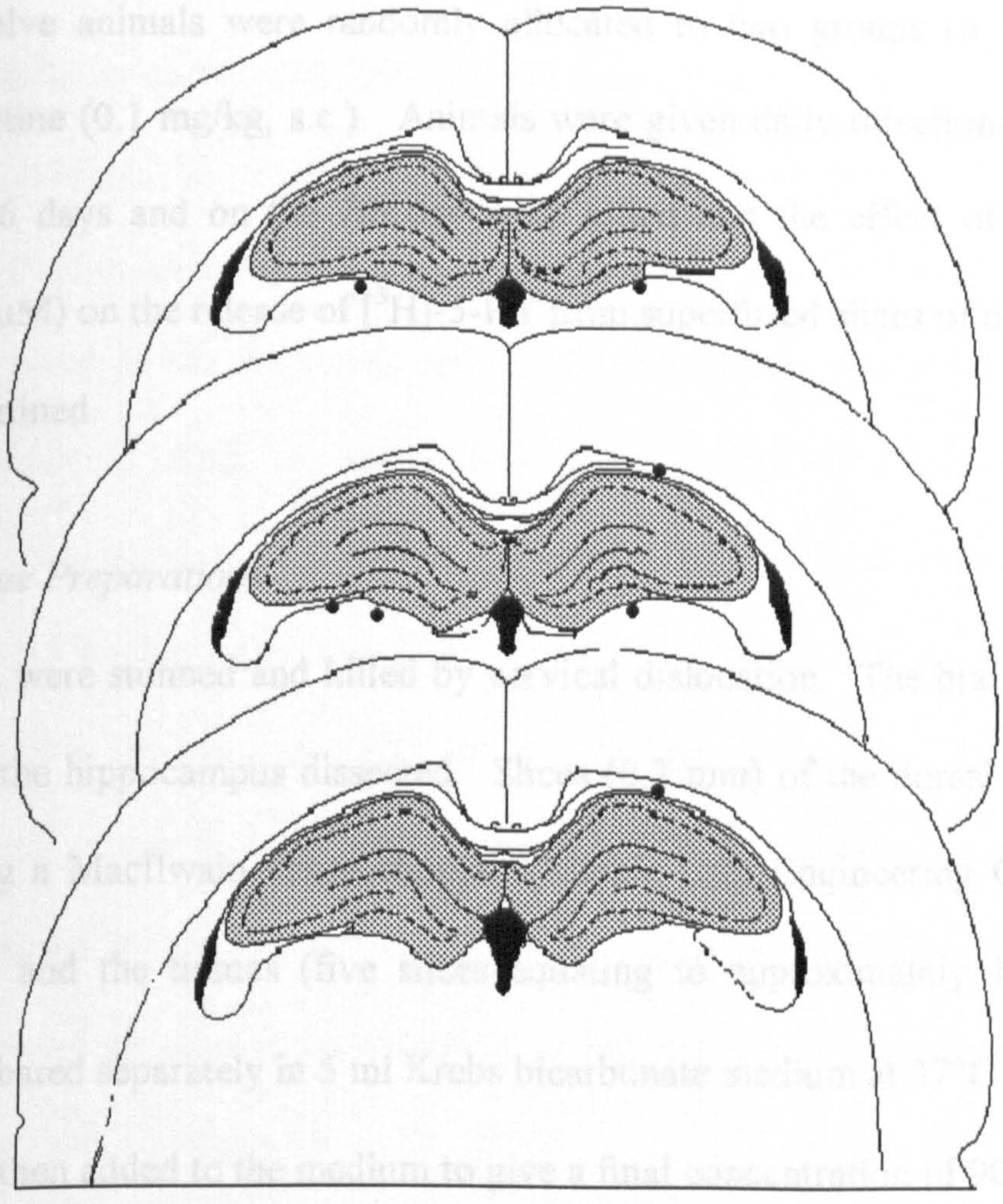

Figure 8.1 Diagrammatic representation of coronal sections (3.14 to 3.6 mm posterior to bregma) through the rat brain showing the area of placements accepted as falling within the dorsal hippocampus (shaded). Placements falling outside the target area are shown by filled circles marking the tip of the injection needle (data from these rats were excluded from analysis).

Measurement of release

Twelve animals were randomly allocated to two groups (n = 6/group): vehicle or nicotine (0.1 mg/kg, s.c.). Animals were given daily injections of vehicle or nicotine for 6 days and on the 7th day were killed and the effect of nicotine (50, 100 and 200μM) on the release of [^{3}H]-5-HT from superfused slices of dorsal hippocampus was examined.

Tissue Preparation

Rats were stunned and killed by cervical dislocation. The brain was rapidly removed and the hippocampus dissected. Slices (0.2 mm) of the dorsal hippocampus were cut using a MacIlwain tissue chopper (Mickle Lab. Engineering Co., Gomshall, Surrey, UK) and the tissues (five slices equating to approximately 10-15 mg wet weight) incubated separately in 5 ml Krebs bicarbonate medium at 37°C for 10 min. [^{3}H]-5-HT was then added to the medium to give a final concentration of 90nM and the incubation was continued for another 30 min. Following incubation the slices were recovered and transferred to a superfusion chamber.

Superfusion and Release

The hippocampal slices were placed between two filter disks (GF/B) in chambers (0.25 ml volume) of a Brandel SF-06 superfusion system and superfused with oxygenated (95% O_2 / 5% CO_2) bicarbonate medium at a rate of 0.5 ml/min for 30 min to obtain a consistent resting rate of fractional release. Following this washout period, samples of the superfusate were collected every 2 min. The effect of nicotine on the release of

[^{3}H]-5-HT was measured by exposing the tissue slices to the drug for 2 min. At the end of the experiment, the tissue slices were dissolved in 1 ml soluene (Packard) followed by addition of 200μM, glacial acetic acid (Sigma) and 3 ml scintillation fluid (Ultima Gold-XR; Packard). The radioactivity in each fraction was measured by liquid scintillation counting (LKB Wallac, 1214 Rackbeta). The efflux of radioactivity was expressed as the fractional rate coefficient (FRC = radioactivity released during a particular fraction, expressed as a percentage of the total amount of radioactivity present in the slices at that time). Basal resting release was taken as the mean FRCs of the two fractions prior to drug superfusion (S1), while nicotine-evoked release was defined as the maximum FRC after drug superfusion (S2). Nicotine-evoked increases in basal release was determined by statistically analysing the magnitude of S2 values compared to S1 values. In Figure 8.3 all nicotine-evoked increases in release are expressed as percentage increases above baseline, calculated by determining the % increase in S2 values above S1 values.

Statistics

The scores from the behavioural tolerance study were analysed with two-way ANOVA with chronic treatment and acute hippocampal injection as the two factors. Comparisons with individual groups were then made with Fisher's post-hoc tests, and it is the significances of these that are shown in the figures and the tables.

For the measurement of release, the increase from the baseline of the nicotine-evoked release was analysed by single factor repeated measures for each dose of nicotine. The

difference between the chronic vehicle and nicotine groups was analysed by two-way ANOVA with chronic treatment and dose of nicotine as the two factors. Comparisons between the individual groups were then made with Fisher's post-hoc tests.

8.3 Results

Effect of a dorsal hippocampal injection of nicotine after chronic nicotine treatment

Figure 8.2 shows that chronic nicotine treatment significantly modified the response to nicotine when directly administered into the dorsal hippocampus in the social interaction test [chronic x acute interaction: $F(1,28)=4.0$, $p=0.05$]. Thus, acute administration of nicotine (1μg) into the dorsal hippocampus significantly decreased the time spent in social interaction, and tolerance was seen to this effect following pre-treatment for 6 days with nicotine (0.1 mg/kg; s.c.). Although there was some reduction in locomotor activity with acute nicotine administration in the vehicle pre-treated rats, there was no overall significant effect of either acute or chronic nicotine treatment after dorsal hippocampal administration on locomotor activity [$F(1,28)=2.4$, $F(1,28)=1.0$, respectively] or rears [$F(1,28)=0.6$, $F(1,28)=0.5$, respectively], see Table 8.1.

Effect of nicotine on rat dorsal hippocampal [^{3}H]-5-HT release after chronic nicotine treatment

Nicotine stimulated a significant dose-related increase in [^{3}H]-5-HT release from dorsal hippocampal slices, but this effect was significantly lower in slices taken from rats that had been chronically treated with nicotine [$F(1,31)=20.6$, $p<0.01$], indicating

the development of tolerance to the effects of nicotine on 5-HT release, see Figure 8.3. There were no significant differences between the baseline levels of [^{3}H]-5-HT release for all the animals, which ranged from 0.89-0.96 frc.

Table 8.1 Mean (± sem) locomotor activity (beam breaks) of rats pre-treated for 6 days with vehicle or nicotine (0.1 mg/kg, s.c.) and then on the 7th day challenged with bilateral injection into the dorsal hippocampus with aCSF or nicotine (1μg). Rats were tested in the high light familiar (HF) test condition, 3 min after central injection.

	Locomotor Activity
Pre-treatment: Vehicle	
aCSF (n=9)	298.3 ± 20.5
1μg (n=7)	233.9 ± 25.6
Pre-treatment: Nicotine (0.1 mg/kg, s.c.)	
aCSF (n=9)	301.4 ± 26.9
1μg (n=7)	313.1 ± 31.9

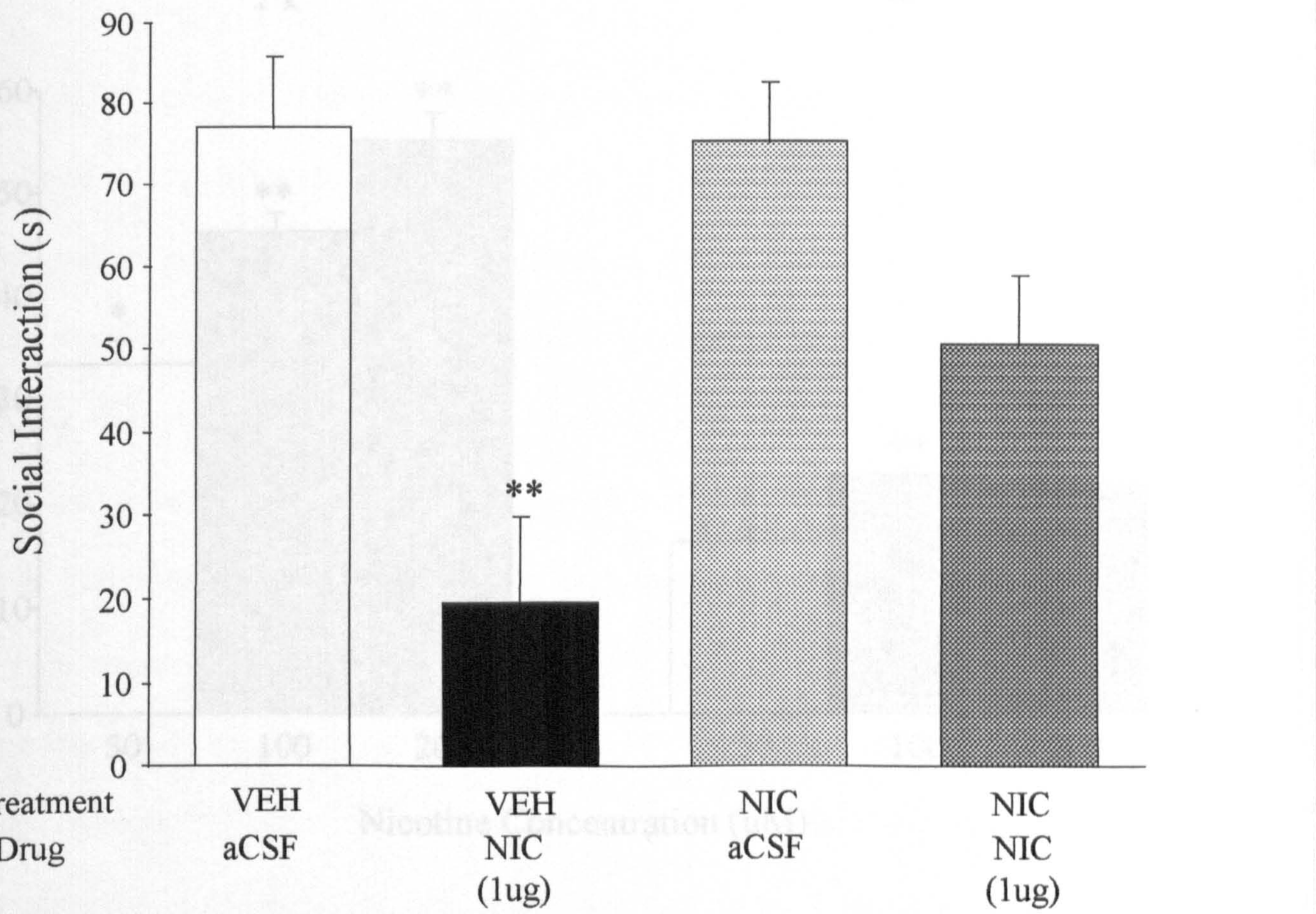

Figure 8.2 Mean (± sem) time (s) spent in social interaction by rats that had been treated for 6 days with vehicle or nicotine (0.1 mg/kg, s.c.) and then challenged with bilateral injection into the dorsal hippocampus with either aCSF or nicotine (1μg). Rats were tested in the high light familiar (HF) test condition, 3 min after hippocampal injection. ** $p<0.01$ compared with all other groups.

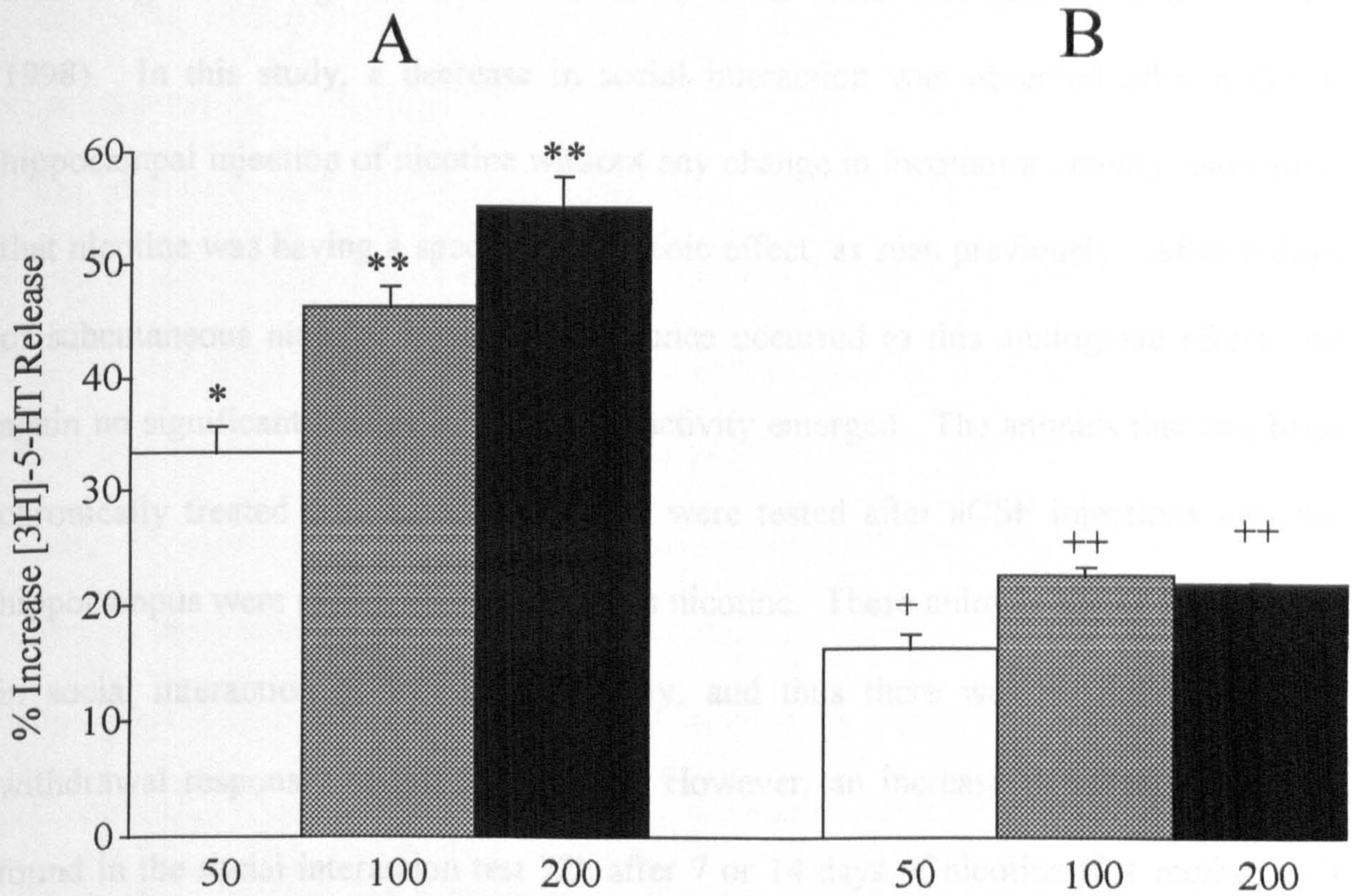

Figure 8.3 Mean (± sem) % increase in basal [3H]-5-HT release evoked by nicotine (50, 100 and 200μM) from rat dorsal hippocampal slices from rats that had been treated for 6 days with vehicle (A) or nicotine (0.1 mg/kg, s.c.; B). * $p<0.05$ and ** $p<0.01$ compared with the baseline release, and $^{+}$ $p<0.05$ and $^{++}$ $p<0.01$ compared with both the baseline release and the nicotine evoked release in vehicle treated animals.

8.4 Discussion

Previous studies established the importance of the dorsal hippocampus as one area mediating the anxiogenic effect of nicotine in the social interaction test (File et al., 1998). In this study, a decrease in social interaction was observed after a dorsal hippocampal injection of nicotine without any change in locomotor activity, indicating that nicotine was having a specific anxiogenic effect, as seen previously. After 6 days of subcutaneous nicotine injections, tolerance occurred to this anxiogenic effect and again no significant change in locomotor activity emerged. The animals that had been chronically treated with nicotine but that were tested after aCSF injections into the hippocampus were in 24h withdrawal from nicotine. These animals showed no change in social interaction or locomotor activity, and thus there was no indication of a withdrawal responses at this time-point. However, an increase in anxiety has been found in the social interaction test 72h after 7 or 14 days of nicotine (0.1 mg/kg; s.c.) treatment (Chapter 2). Thus, the results of the present study demonstrate that tolerance can be observed in the social interaction test at a time-point at which no withdrawal responses are found. An oppositional mechanism of tolerance is one that involves the progressive recruitment of processes that oppose the acute effect of the drug. Thus, following withdrawal of the drug these processes work unopposed and a behavioural response is seen in the opposite direction of the acute drug effect (Young and Goudie, 1995). As no withdrawal response was observed it suggests that the underlying mechanism is unlikely to be an oppositional one.

The present results demonstrate that the dorsal hippocampus is an area crucially concerned with mediating the development of tolerance to the anxiogenic effect of nicotine. Nicotinic acetylcholine receptors (nAChRs) are situated predominantly on presynaptic terminals in the brain where they modulate the release of many neurotransmitters, including 5-HT (Vizi and Kiss, 1998; Wonacott, 1997). Previously it has been suggested that the anxiogenic effect of nicotine in the dorsal hippocampus is mediated by nicotine's action at heteroreceptors on the 5-HT terminals, causing an increase in 5-HT release (File et al., 2000b) and an activation of post-synaptic 5-HT_{1A} receptors, because the 5-HT_{1A} receptor antagonist WAY 100,635 can reverse the anxiogenic effect of nicotine (Kenny et al., 2000b). After 6 days of nicotine pre-treatment there was a significant attenuation of the ability of nicotine to stimulate 5-HT release in the dorsal hippocampus, which could have arisen as a result of receptor desensitisation (Radcliffe et al., 1999). This decreased effect of nicotine on the release of 5-HT from the terminals could be sufficient to mediate tolerance to the anxiogenic effect observed after nicotine injection into the dorsal hippocampus, and could account for the development of tolerance without the incidence of a withdrawal response.

Rather high concentrations of nicotine are needed to evoke 5-HT release from dorsal hippocampal slices and the nAChRs are more sensitive to the nicotinic agonists, dimethylphenylpiperazine and lobeline, than to nicotine (Lendvai et al., 1996). However, there are two factors that are likely to enhance this effect in vivo, especially in conditions that evoke anxiety. With an increase in anxiety, it has been shown that there is an increase in 5-HT and glycine release in the dorsal hippocampus (File et al.,

1987b) and both these factors will enhance nicotine's effects on 5-HT release (File et al., 2000b). The effects of nicotine on 5-HT release are most likely to be seen in vivo following high systemic doses of nicotine (0.5 and 1.0 mg/kg) and by 1μg injected directly into the dorsal hippocampus, all of which have been shown to have anxiogenic effects (File et al., 1998).

Previous studies have shown that a relatively short period of treatment with a low dose of nicotine is sufficient to lead to the development of tolerance to both the anxiogenic and anxiolytic effects of systemic nicotine (Chapter 2), and to the anxiolytic effects of central injections of nicotine (Chapter 7). The present study has shown that tolerance can also be seen to the anxiogenic effect of central injections of nicotine after the same chronic nicotine treatment regime. At present, it is not clear whether the mechanism and brain regions mediating the anxiogenic effects of high doses (0.5 and 1.0 mg/kg) are the same as those mediating the anxiogenic effect observed 5 min after the 0.1 mg/kg dose. In order to determine this, further experiments are needed, in which antagonists are injected into specific brain regions, following systemic injections of nicotine. So far, both the dorsal hippocampus and the lateral septum have been identified as areas mediating anxiogenic effects (Cheeta et al., 2000a, b; Kenny et al., 2000b; Ouagazzal et al., 1999a, b; File et al., 1998).

In summary, the results from the present series of experiments suggest that changes in the dorsal hippocampus mediate the development of tolerance to the anxiogenic effects of nicotine. A possible mechanism for the tolerance that is seen is an attenuation of the

ability of nicotine to stimulate 5-HT release in the dorsal hippocampus after 6 days of nicotine treatment.

CHAPTER 9

Tolerance to midazolam's anxiolytic effects in the social interaction test after short-term nicotine treatment

9.1 Introduction

Benzodiazepines are still widely used as anxiolytics, despite concerns about the development of dependence (Argyropoulos and Nutt, 1999). In a clinical situation it is difficult to determine whether tolerance develops to their anxiolytic effects, but in several animal tests of anxiety it has been shown to develop after about 3 weeks of treatment (Fernandes and File, 1999; Fernandes et al., 1999; Chopin et al., 1993; File et al., 1987a; Treit, 1985; Vellucci & File, 1979). Nicotine has been shown to have anxiolytic effects in non-smoking young women exposed to mild stress (File et al 2000a; Netter et al 1998) and in several animal tests (Chapter 2; File et al., 1998; Cao et al., 1993; Brioni et al., 1993; Costall et al., 1989b; Vale and Green, 1986). However, in contrast to the benzodiazepines, tolerance develops much more quickly. After 6 days of treatment with nicotine (0.1 mg/kg/day; s.c.) tolerance develops to the anxiolytic effects in the social interaction test after been probed on the 7th day with either a subcutaneous or central injection of nicotine (Chapters 2 and 7).

It is possible that many smokers will be receiving benzodiazepine medication as there is an increased incidence of anxiety disorders among smokers (Breslau et al., 1991), and 64% of adolescent girls say smoking makes them feel calmer (McNeill et al.,

. 1997). The purpose of the present study was therefore to determine whether a short period of nicotine pre-treatment modified the anxiolytic effects of benzodiazepines. We decided to probe the anxiolytic efficacy of the benzodiazepines after direct administration to the brain, since there is evidence of a possible pharmacokinetic interaction between chronic nicotine and benzodiazepines (Smith et al., 1983; Norman et al, 1981). The dorsal hippocampus and the DRN are two brain sites that mediate the anxiolytic effects of benzodiazepines (Nazar et al., 1999; Gonzalez et al., 1998; Gonzalez and File, 1997; Stefanski et al., 1993; Thiebot et al., 1982). The effects of nicotine on anxiety are somewhat different in these two regions. Low doses are anxiolytic in the DRN, but silent in the dorsal hippocampus, and high doses are anxiogenic in both regions (Chapter 7; Cheeta et al., 2000a; File et al., 1998). After one week of nicotine treatment there is tolerance to the anxiolytic effects in the DRN and to the anxiogenic effects in the dorsal hippocampus (Chapters 7 and 8). These two brain areas were selected to probe the efficacy of midazolam in the social interaction test of anxiety, after one week of nicotine pre-treatment.

This study also investigated whether there were any changes in benzodiazepine receptor binding in these brain areas as a result of chronic nicotine treatment. The binding was conducted at two concentrations in order to obtain an estimate of whether any changes were in receptor affinity or density.

9.2 Materials and Methods

Animals

Male hooded Lister rats (Charles River, Margate, Kent, UK) weighing between 220-250g were housed singly. Rats that had undergone surgery were allowed to recover for 4 days prior to the start of chronic injections. Food and water were freely available, and the room in which they were housed was lit with dim light and maintained at 22°C. Lights were on from 0700-1900h.

Apparatus

The social interaction test, see Chapter 2 for description.

Surgery

Surgery was conducted as described in Chapters 3 and 7.

Drugs and chemicals

For the chronic sub-cutaneous injections, (-)-nicotine hydrogen tartrate was dissolved in distilled water, in a volume of 1 ml/kg body weight and a dose of 0.1 mg/kg was used; control animals received equal volume injections of distilled water. All doses are given as free base. For the intracerebral injections midazolam maleate (kindly donated by Roche products Ltd, Welwyn Garden City, UK) was dissolved in 0.9% sodium chloride (NaCl) solution. Injections were 0.5µl, and were made over a period of 30s

using a CMA/102 microdialysis pump and the needles were left in position a further 30s to allow drug diffusion; control animals received 0.5µl infusions of 0.9% NaCl.

[^{3}H]Flunitrazepam (85 Ci/mmol; DuPont, NEN, Stevenage, UK) was diluted to the appropriate concentration using ice cold 50mM Tris HCl. Diazepam (Sigma, UK) was made up as a 1 mg/ml stock solution by dissolving in ethanol and then diluted to the appropriate concentration using ethanol. 50mM ice cold buffer was made from Tris HCl and Tris base purchased from Sigma (Poole, Dorset, UK).

Behavioural Testing

Animals were randomly allocated to a 6 day pre-treatment with either vehicle (n=52) or nicotine (0.1 mg/kg, s.c.; n=43) for 6 days. On the 7th day, no s.c. injections were given and within each of the two chronic treatment groups, animals were randomly allocated to the following dorsal hippocampal injection groups: 0.9% NaCl; midazolam (2µg, 4µg or 8µg), and to the following DRN injection groups: 0.9% NaCl; midazolam (4µg or 8µg). The numbers of rats in each group after verification of cannula placements are shown in Table 9.1. On the test day each operated animal was allocated to an unoperated/undrugged test partner that was matched in weight (± 10g).

In order to familiarise the rats with the social interaction test arena, each rat was placed singly in the test arena, under high light conditions of illuminance (300 lux) arena, on the 2 days prior to testing, for a 5 min familiarisation trial. In both experiments, the operated rat was placed together with its unoperated partner in the test

arena, three minutes after central injection, and only social interaction initiated by the operated animal was scored. Social interaction was scored for 4.5 min by an observer blind to the drug treatment. Rats were tested between 0900 and 1230h in an order randomised for drug treatment, and at the end of each trial the arena was wiped with a damp cloth and faecal pellets removed.

Histology

At the end of behavioural testing all animals were sacrificed, the brains removed and the injection sites verified histologically (according to the atlas of Paxinos & Watson, 1986) by a person blind to drug treatment. Figures 9.1 and 9.2, depicting coronal slices through the DH and DRN, show the target site and the positions of the tips of the injection needles for the rats excluded from statistical analysis because of placement errors.

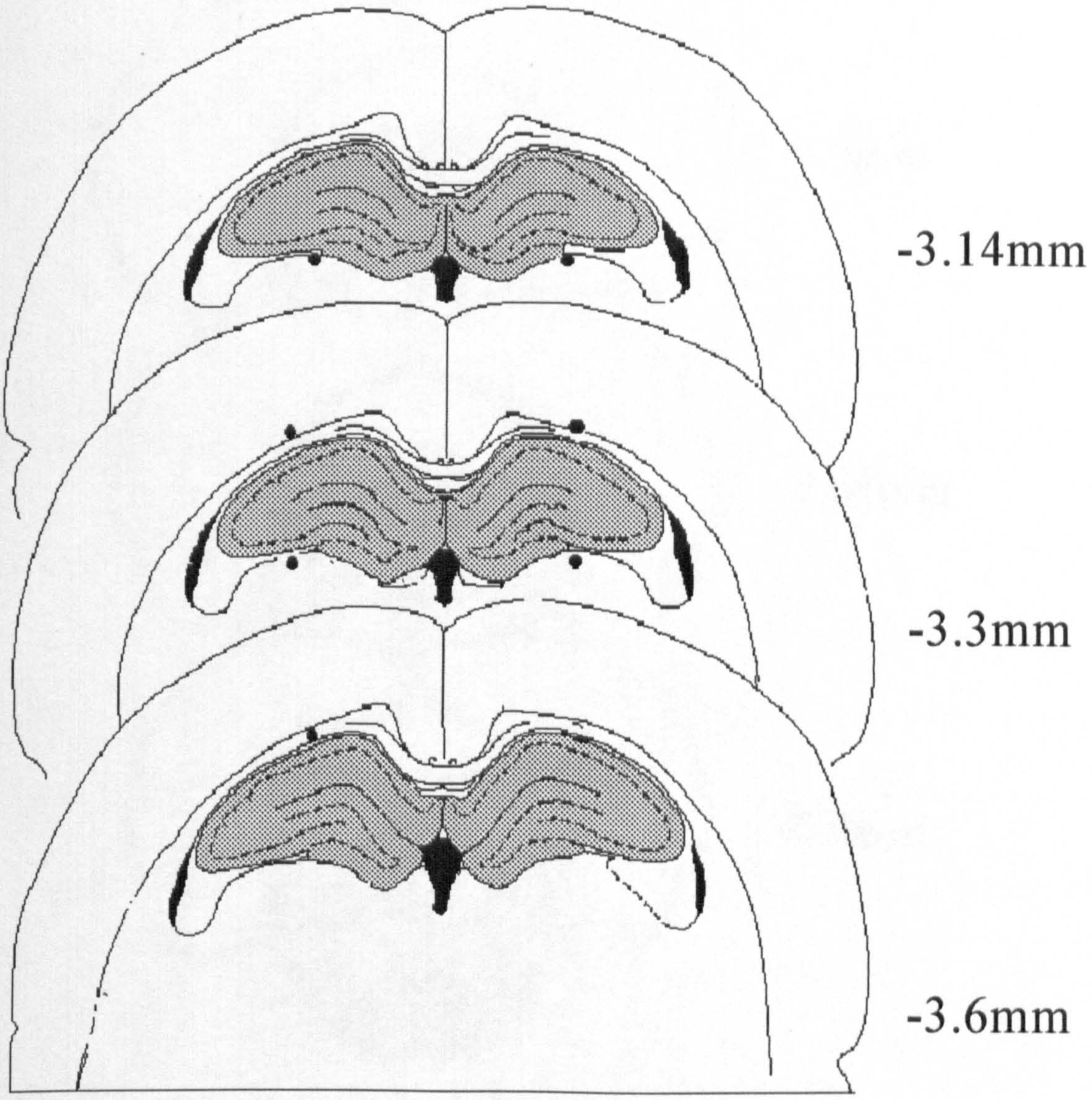

Figure 9.1 Diagrammatic representation of coronal sections (3.14 to 3.6 mm posterior to bregma) through the rat brain showing the area of placements accepted as falling within the DH (shaded). Placements falling outside the target area are shown by filled circles marking the tip of the injection needle (data from these rats were excluded from analysis).

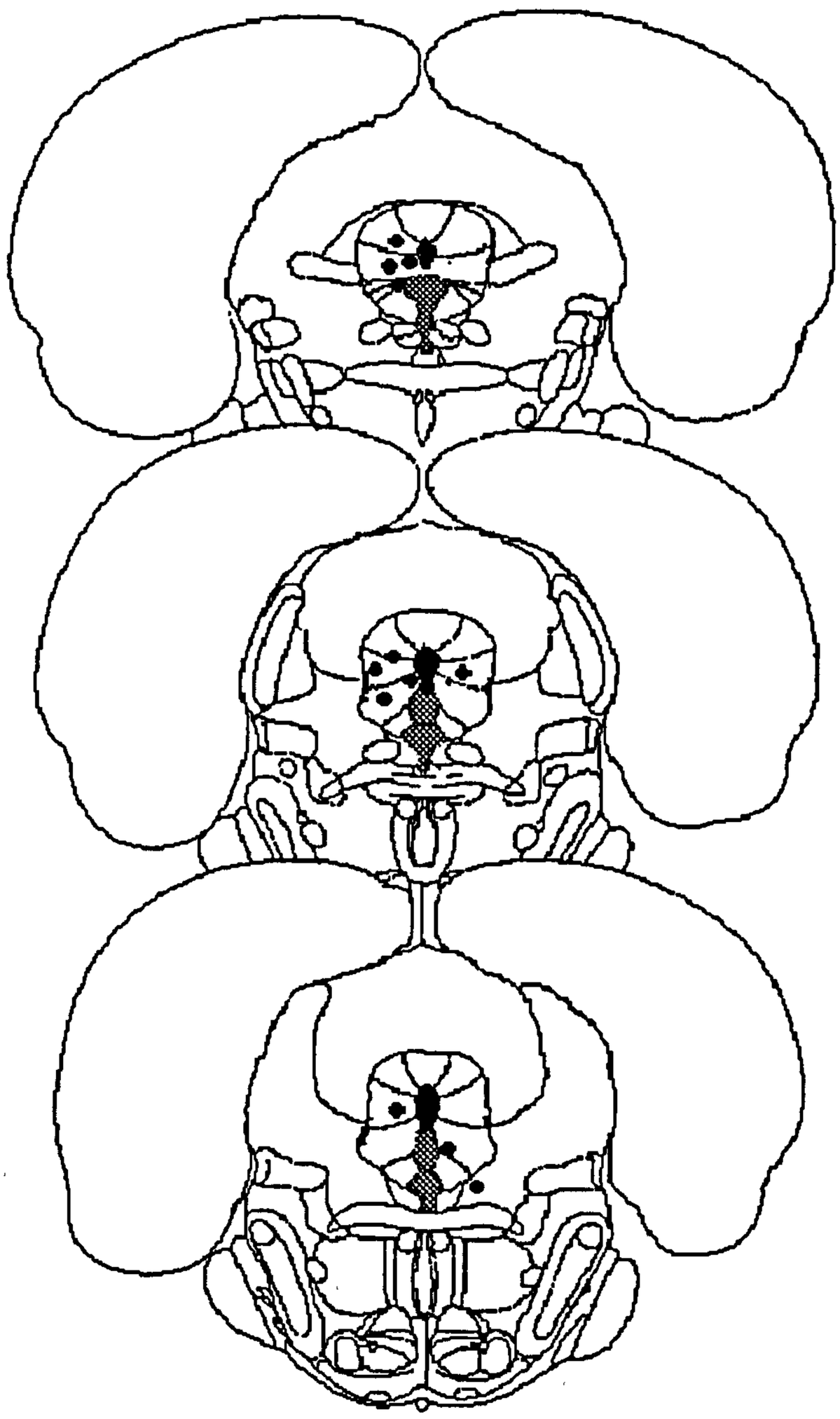

Figure 9.2 Diagrammatic representation of coronal sections (7.3 to 7.8 mm posterior to bregma) through the rat brain showing the area of placements accepted as falling within the DRN (shaded). Placements falling outside the target area are shown by filled circles marking the tip of the injection needle (data from these rats were excluded from analysis).

Measurement of binding

Animals were randomly allocated to treatment for 7 days with either vehicle or nicotine (0.1 mg/kg/day; s.c.). Animals were sacrificed 30min after their last injection by decapitation and their brains removed, and the hippocampus (vehicle, n=6; nicotine, n=5) and mid-brain (vehicle, n=6; nicotine, n=7) dissected. These brain regions were frozen in an isopentane dry ice bath and stored in a -80°C freezer until needed.

Membrane Homogenate Preparation

Frozen brain tissue was defrosted, weighed and suspended in 15mls of ice cold distilled water. Each sample was then homogenised using a polytron homogeniser at maximum setting for 10s. The homogenate was then centrifuged at 38000g for 20 min at 4°C. The supernatant was discarded and the pellet was resuspended in 15 mls of 50mM Tris HCl and homogenised. The homogenate was then centrifuged at 38000g for 30 min at 4°C. This step was repeated a further three times. The final sample was then suspended in 10mls of 50mM Tris HCl and frozen. When needed, the sample was defrosted and the volume made up to 15mls and re-spun at 38000g for 30 min at 4°C.

[^{3}H]Flunitrazepam Binding Assay

Aliquots (75µg protein/ml final concentration) of membrane suspension were incubated with either 2 or 10 nM [^{3}H]flunitrazepam, corresponding to the K_D and B_{MAX} concentrations respectively, in a final volume of 0.5ml for 1h on ice. Non-specific binding was determined in the presence of 3µM diazepam. Specific and non-specific

binding were both performed in triplicate. Following incubation, the samples were filtered by vacuum filtration through Whatman GF/B glass fibre filters using a Brandel cell harvester (Semat, Hertfordshire, UK) and washed 3 times with 4mls ice cold 50mM Tris HCl in order to separate bound from free ligand. The filter paper was pre-soaked in 0.05% polyethylenimine to inhibit non-specific binding. The filters were then put into scintillation vials with 4mls of Packard Emulsifier safe scintillation fluid and mixed. The radioactivity of the samples was measured using liquid scintillation counting (LKB Wallac, 1214 Rackbeta) and the amount of specific binding of ligand determined (as fmol/mg protein) from this.

Protein Assay

Protein concentrations were assessed in duplicate using Comassie Plus protein assay reagent (Pierce) and measuring absorbance at 595nm. Bovine serum albumin was used as the protein standard.

Statistics

The behavioural scores were analysed with two-way analyses of variance (ANOVA) with chronic treatment and acute DRN or DH injections as the two factors. Comparisons with individual groups were then made with least significance difference (LSD) post-hoc tests, and it is the significances of these that are shown in the figures and the tables. The specific binding (fmol/mg protein) was analysed by one-way ANOVAs and it is the significances of these that are shown in the figures.

9.3 Results

Effects of midazolam in the DH and DRN

It can be seen from Figures 9.3 and 9.4 that microinjection of midazolam into the DH and DRN significantly ($p<0.01$) increased social interaction in the vehicle-pre-treated rats. There were no changes in locomotor activity (see Table 9.1) and thus midazolam had clear anxiolytic effects in both brain regions. The nicotine pre-treatment significantly modified these anxiolytic effects ($p<0.01$ in both cases) and only 4μg midazolam in the DRN retained any anxiolytic efficacy.

Effects on [^{3}H]Flunitrazepam binding

Nicotine treatment significantly decreased [^{3}H]flunitrazepam binding in the DH at both 2 and 10 nM ($p<0.05$, in both cases), see Figure 9.5. The ratio of binding seen at both 2 and 10 nM in nicotine pre-treated animals was not significantly different ($p>0.05$) to that seen in vehicle pre-treated animals, suggesting it is most likely that the decreased binding is due to a decrease in receptor density. Nicotine treatment did not change [^{3}H]flunitrazepam binding in the midbrain at either concentration, see Figure 9.5.

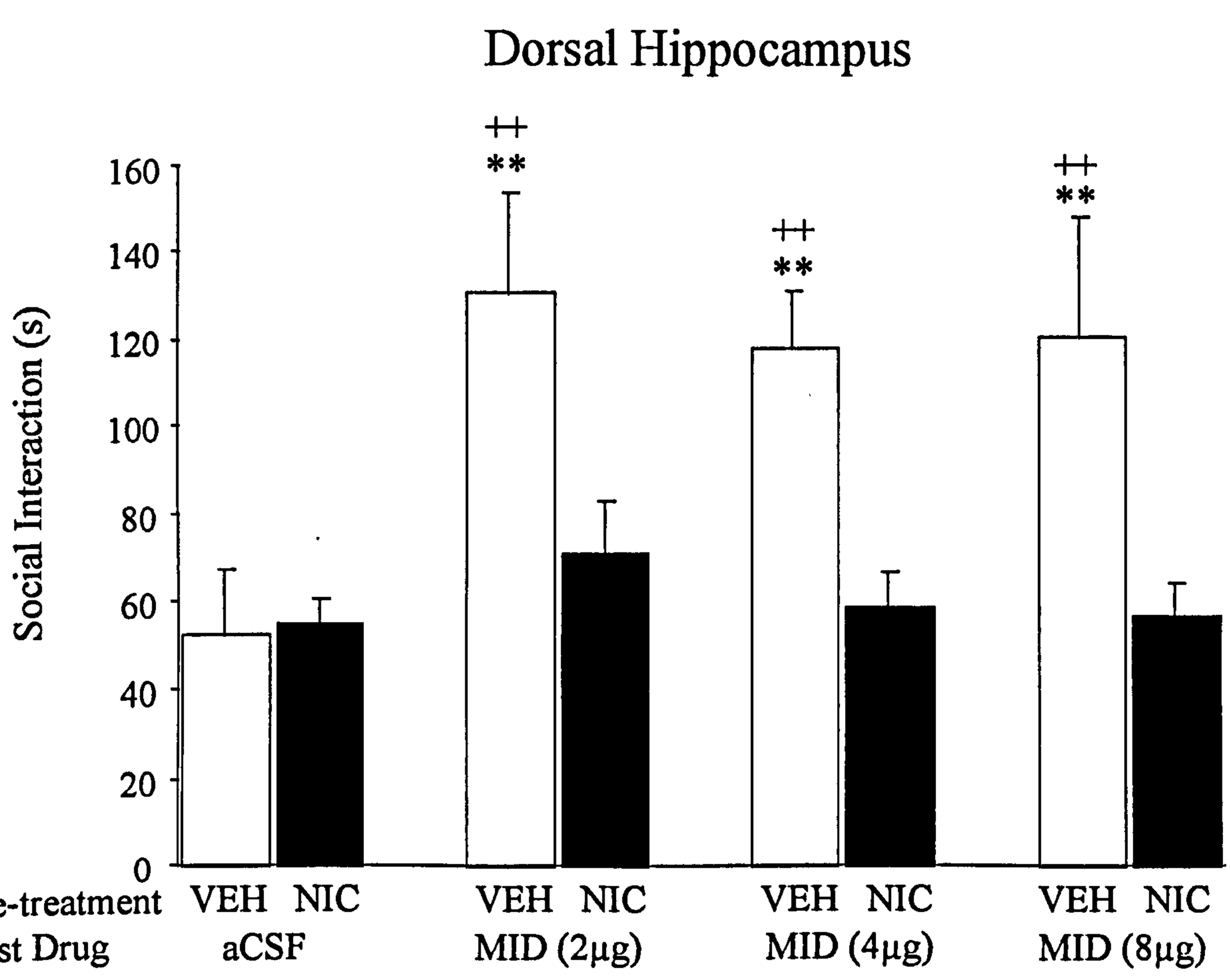

Figure 9.3 Mean (± sem) time (s) spent in social interaction by rats that had been treated for 6 days with vehicle or nicotine (0.1 mg/kg, s.c.) and then challenged with bilateral injection into the DH with either 0.9% NaCl or midazolam (2μg, 4μg and 8μg). Rats were tested in the high light familiar (HF) test condition, 3 min after hippocampal injection. ** $p<0.01$ compared with the vehicle control, $^{++}$ $p<0.01$ compared with animals treated chronically with vehicle for 6 days and then challenged with 2μg, 4μg and 8μg midazolam in the DH on the 7th day.

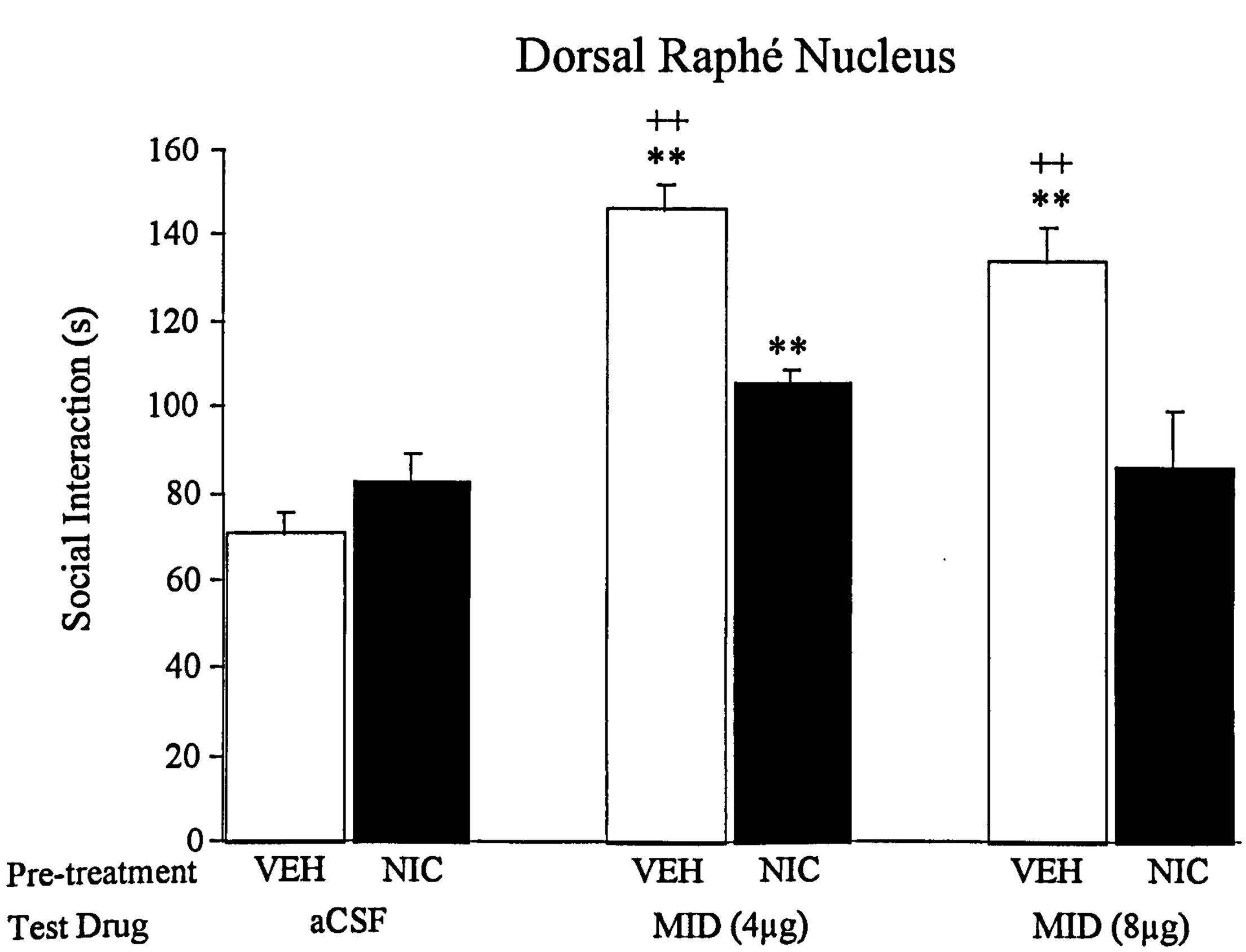

Figure 9.4 Mean (± sem) time (s) spent in social interaction by rats that had been treated for 6 days with vehicle or nicotine (0.1 mg/kg, s.c.) and then challenged with unilateral injection into the DRN with either 0.9% NaCl or midazolam (4µg and 8µg). Rats were tested in the high light familiar (HF) test condition, 3 min after DRN injection. ** $p<0.01$ compared with the vehicle control, $^{++}$ $p<0.01$ compared with animals treated chronically with vehicle for 6 days and then challenged with 4µg and 8µg midazolam in the dorsal hippocampus on the 7th day.

Table 9.1 Mean (± sem) locomotor activity (beam breaks) made by rats that had been treated for 6 days with vehicle or nicotine (0.1 mg/kg, s.c.) and then challenged with bilateral injection into the DH with either 0.9% NaCl or midazolam (2μg, 4μg and 8μg), or unilateral injection into the DRN with either 0.9% NaCl or midazolam (4μg and 8μg). Rats were tested in the high light familiar (HF) test condition, 3 min after injection. The numbers in parentheses are the number of animals/group after verification of the cannula placements. nd = no data.

	Locomotor Activity	
	DH	DRN
Pre-treatment: Vehicle		
0.9% NaCl	190.9 ± 25.0 (7)	280.6 ± 20.9 (14)
2μg	254.4 ± 16.9 (5)	nd
4μg	209.9 ± 28.2 (6)	259.7 ± 20.1 (6)
8μg	216.2 ± 23.5 (5)	260.1 ± 9.1 (9)
Pre-treatment: Nicotine (0.1 mg/kg, s.c.)		
0.9% NaCl	191.6 ± 18.0 (5)	285.6 ± 24.7 (8)
2μg	196.5 ± 12.5 (6)	nd
4μg	251.0 ± 33.4 (6)	294.4 ± 17.5 (7)
8μg	297.0 ± 28.3 (6)	247.8 ± 19.6 (5)

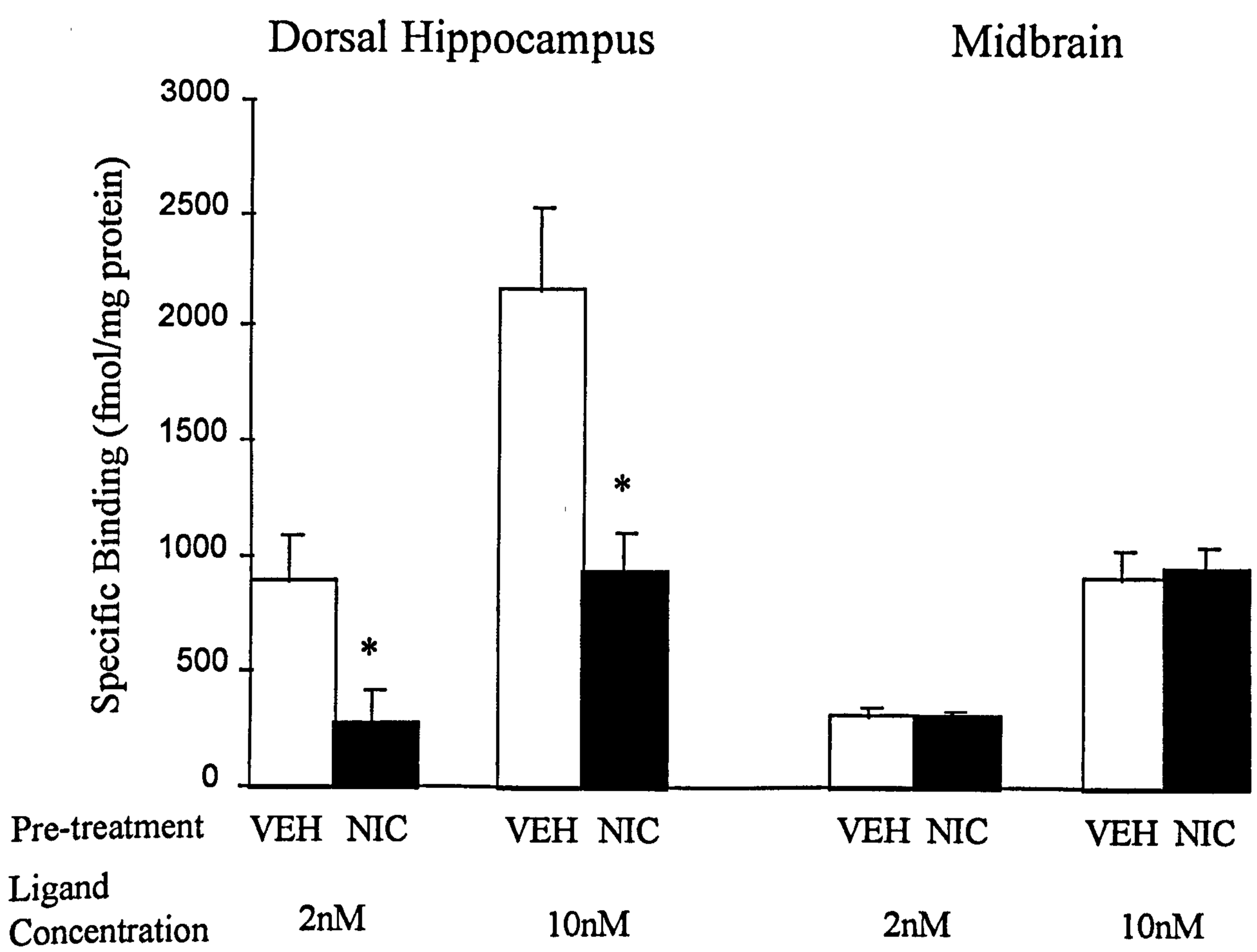

Figure 9.5 Mean (± sem) specific binding (fmol/mg protein) of [^{3}H]-Flunitrazepam binding to dorsal hippocampal and midbrain membranes of rats that had been treated for 7 days with vehicle or nicotine (0.1 mg/kg, s.c.). *$p<0.05$ compared with vehicle control.

9.4 Discussion

The present study demonstrated that, in the social interaction test, administration of midazolam to the DH and DRN had significant anxiolytic effects in vehicle pre-treated animals. These anxiolytic effects were not dose-dependent, but this was probably due to the supra-maximal doses of midazolam that were used. These high doses were used in order to investigate if there was a shift in the anxiolytic effects of midazolam after 6 days of nicotine treatment. However, after 6 days of nicotine (0.1 mg/kg/day; s.c.) treatment tolerance developed to these anxiolytic effects of midazolam in both the DH and DRN. This rate of cross-tolerance development is much more rapid than the tolerance that develops after chronic benzodiazepine treatment, which usually takes around 3 weeks to develop (Fernandes and File, 1999; Fernandes et al., 1999; Chopin et al., 1993; File et al., 1987a; Treit, 1985; Vellucci & File, 1979). There is considerable evidence of cross-tolerance between nicotine and ethanol (Luo et al, 1994; de Fiebre et al, 1993; Collins et al, 1988) and thus the cross-tolerance seen in this study is not surprising given the similarity in the pharmacological profile of benzodiazepines and ethanol.

Since central administration of benzodiazepines was used a pharmacokinetic explanation of the results was able to be excluded. O'Neill and Brioni (1994) have shown that the acute anxiolytic effect of nicotine can be blocked by the benzodiazepine receptor antagonist, flumazenil, which suggests some interaction between nicotine and benzodiazepine receptors in modulating anxiety. Certainly, the reduction in benzodiazepine binding found in the hippocampus would be sufficient to explain the

behavioural tolerance seen after microinjections of midazolam into this region. No decrease in binding in the midbrain was found, but this may be due to the relatively gross dissection of this region, of which the dorsal raphé nucleus forms only a small part.

The mechanism by which nicotine administration changes benzodiazepine binding is at present unknown, but one possibility is via a nicotine-induced increase in GABA release. Nicotine increases GABA release in the hippocampus (Lu et al., 1998; Alkondon et al., 1997). A decrease in benzodiazepine receptor binding could be an adaptive change to the increased GABA release following repeated nicotine injections. Lu et al (1998) also found that nicotine increased GABA release in synaptosomes from the midbrain, but this effect was less marked than that seen in synaptosomes from the hippocampus. It is therefore possible that a different explanation must be sought for the tolerance found to the effects of midazolam injected into the DRN.

Nicotine increases 5-HT release in the DRN (Mihailescu et al., 1998) and its anxiolytic effect in the DRN can be reversed by the 5-HT_{1A} receptor antagonist, WAY 100,635 (Chapter 7). This suggests that the anxiolytic action of nicotine is mediated by stimulation of the 5-HT_{1A} autoreceptors. A stimulation of the autoreceptors would lead to a reduction in raphé firing rate and, indeed, intravenous nicotine has been shown to reduce raphé firing, an effect that was blocked by WAY 100,635 (Engberg et al., 2000). After chronic treatment, nicotine administration may no longer change the raphé firing, compared with the control level. The anxiolytic effect of benzodiazepines

is also thought to be mediated by a reduction in raphé firing rate and hence in 5-HT release in the terminal areas (Kahn et al., 1988; Balfour, 1980). Hence the administration of midazolam into the DRN would simply have substituted for the daily dose of nicotine. In the present experiment we tested the rats 24h after their last nicotine injection, a time at which a withdrawal response has not previously been observed in the social interaction test (Chapters 2 and 7) and was not observed in the present experiment. An increase in anxiety in this test is found 72h after nicotine withdrawal (Chapter 2) and during nicotine withdrawal there is enhanced sensitivity to the inhibitory effect of 8-OH-DPAT on DRN firing (Rasmussen & Czachura, 1997). This suggests the increased anxiety during nicotine withdrawal results from increased DRN firing and subsequent 5-HT release in limbic areas. Thus, we would expect that benzodiazepines would be as effective as nicotine at reversing this nicotine withdrawal response, although one clinical study has shown this not to be case (Hao et al., 1988). However, in this study a number a measures were looked at and thus any effect on anxiety may have been obscured.

Whilst further studies are needed into the roles of benzodiazepine receptors, GABA-benzodiazepine receptor coupling and the 5-HT system in mediating cross-tolerance between the benzodiazepines and nicotine, there are clear clinical implications of these results. The results of this study suggest that the clinical efficacy of benzodiazepines may be reduced in smokers. This is particularly pertinent since patients dependent on benzodiazepines are significantly more likely to smoke than people who are not (Lekka et al., 1997).

CHAPTER 10

Discussion

10.1 Differences Between the Tests of Anxiety

There is increasing evidence that suggests animal tests of anxiety measure different types of anxiety. Several factor analysis studies have provided evidence that different animal tests of anxiety are reflecting different underlying factors and hence may be modelling different anxiety disorders (Flaherty et al., 1998; Ramos et al., 1997; Belzung and Pape, 1994; File, 1992). Furthermore, accumulating evidence from lesion and central drug administration has shown that different brain regions and neurotransmitters control behaviour in the different animal tests of anxiety (Menard and Treit, 1999). Differential effects have also been found following systemically administered drugs (Treit et al, 1993; Fernandez-Guasti et al, 1999) and after the stress of inescapable shock (Steenbergen et al, 1990). The social interaction test has been extensively validated as a model of GAD (File 1997, 1980), whereas it has been suggested that the elevated plus-maze may model some aspects of panic disorder (Graeff et al., 1993). It can be seen from Table 10.1 that the pattern of nicotine's effects on anxiety after acute and chronic nicotine administration, and after withdrawal from chronic nicotine differs in the social interaction and elevated plus-maze tests, thus providing further evidence for the difference in anxiety generated between the two tests, and suggesting that nicotine may have differential effects in different anxiety disorders.

Table 10.1 The effects of nicotine (0.1 mg/kg; s.c.) in the social interaction and plus-maze tests after acute and chronic (7 days) treatment, and withdrawal from chronic treatment. ↓, indicates a significant anxiogenic effect; ↑, indicates a significant anxiolytic effect; =, indicates no effect on anxiety.

Time after nicotine	Social Interaction	Elevated Plus-maze
Acute		
5 min	↓ (0.1 & 0.45)	= (0.05 - 0.45)
30 min	↑ (0.05 - 0.25) ↓ (0.45)	↓ (0.1 - 0.45)
60 min	↓ (0.1)	↓ (0.25 - 0.45)
7days		
5 min	= (0.1)	↑ (0.1)
30 min	= (0.1)	= (0.1)
Withdrawal from 7days of nicotine (0.1 mg/kg) treatment		
24 h	=	↓
72 h	↓	= (unpublished data)

In animal tests of anxiety, nicotine has been shown to have both anxiogenic and anxiolytic effects in rats and mice (Ouagazzal et al., 1999a; File et al., 1998; Vale and Green, 1996; Brioni et al., 1994, 1993; Cao et al., 1993; Costall et al., 1989b). Table 10.1 shows a summary of the results found in Chapters 2-4, and it can be seen that both anxiolytic and anxiogenic effects are seen in the social interaction test, whilst only anxiogenic effects were seen in the elevated plus-maze after acute nicotine administration. These results are in concordance with other studies done in the laboratory (Ouagazzal et al., 1999a; File et al., 1998). One of the most striking findings is that nicotine (0.1 mg/kg) has an anxiolytic effect 30 min after injection in the social interaction test (Chapter 2) but an anxiogenic effect in the elevated plus-maze at this time-point (Chapters 3 and 4). This suggests that a different neurotransmitter or neuroanatomical site is activated in the two tests. Both the anxiolytic and anxiogenic effects of nicotine are known to be mediated through the 5-HT system as both effects have been reversed with the 5-HT_{1A} receptor antagonist WAY 100,635. Therefore, the likely cause for the difference in responses is due to different neuroanatomical substrates being activated.

The hippocampus and septal nuclei are two regions of the limbic system that have long been implicated in the control of anxiety (Gray, 1982), but they are not equally important in all animal tests of anxiety. Both these structures play an important role in mediating the anxiogenic effects of nicotine in the social interaction test (Cheeta et al., 2000b; Ouagazzal et al., 1999b; File et al., 1998), whilst the anxiolytic effect is mediated by the DRN (Chapter 7). In general, the dorsal hippocampus does not seem to play an

important role in controlling behaviour on Trial 1 of the elevated plus-maze (Cheeta et al., 2000a; Ouagazzal et al., 1999a; Gonzalez et al., 1998) and indeed administration of a wide range of nicotine doses administered into this area has no effect in the elevated plus-maze (Ouagazzal et al., 1990a), although there is evidence of this region becoming activated in stressful situations (Netto and Guimaraes, 1996; McBlane and Handley, 1994; Titze-de-Almeida et al., 1994). In contrast to the dorsal hippocampus, the lateral septum plays an important role in mediating behaviour in plus-maze naive rats (Cheeta et al., 2000b; Pesold and Treit, 1996). Direct injection of nicotine into this area produces anxiogenic effects (Cheeta et al., 2000b; (Ouagazzal et al., 1990a), although in the social interaction test 30 min after a dose of 0.1 mg/kg nicotine it seems that an anxiolytic site is the most predominant, i.e. the DRN, whereas in the elevated plus-maze it is an anxiogenic site, i.e. the lateral septum. Interestingly, a higher dose of nicotine (0.45 mg/kg) has an anxiogenic effect in both tests 30 min after injection. Thus, with this dose an anxiogenic site is the most predominant in both tests due to a possible alteration in serotonergic dependent function in the anxiolytic and anxiogenic brain structures.

In the elevated plus-maze only anxiogenic effects are seen after acute nicotine administration, see Table 10.1. This is in contrast to the results of Brioni and colleagues (1994, 1993) that have shown anxiolytic effects in this test in both mice and Wistar rats. There are a number of explanations that could clarify these contrasting results. In both of Brioni's studies an increase in total arm entries was observed suggesting that the anxiolytic effect could be due to an increase in locomotor activity and not actually a change in anxiety. Costall et al. (1989b) also showed anxiolytic effects in the light-dark

exploration test after nicotine treatment but again in this test the measure of anxiety is highly contaminated by changes in locomotor activity. Another possible explanation for the differences could be the differences in strain and species of animals. A review of results from many laboratories using the elevated plus-maze has shown that Wistar rats are much more responsive to anxiolytic drugs, whilst the Lister hooded animals show a greater response to anxiogenic drugs (Hogg, 1996). There is evidence to suggest that responses to drugs can vary depending on the housing conditions of the animals (Ahmed et al., 1995; Thielen et al., 1993; Gardner and Guy, 1984). However, the results in Chapter 4 show that the anxiogenic response to nicotine is seen irrespective of housing conditions since anxiogenic effects are seen in both the singly and group housed animals. Furthermore, there was no change in baseline scores between singly and group housed animals, and they were similar to those found in previous experiments with nicotine which also found an anxiogenic effect in singly housed animals (Ouagazzal et al., 1999a). However, the baseline scores of both the rats and mice in Brioni's studies were approximately half those seen in the singly and group housed animals in Chapters 3 and 4. Previous studies have shown that when the baseline scores are low (e.g. 10% open arm entries) a 5-HT_{1A} receptor agonist administered to the dorsal hippocampus has an anxiolytic effect (Menard and Treit, 1998), whereas with higher baseline scores (30%) neither benzodiazepines nor a 5-HT_{1A} receptor agonist have any action (Gonzalez et al., 1998; File et al., 1996b). In contrast, when baseline scores are high an anxiogenic effect can be seen after administration of a 5-HT_{1A} receptor agonist to the lateral septum (Cheeta et al., 2000a), whereas it is without effect if baseline scores are low (Menard and Treit, 1998). Thus, the differences seen in the response to nicotine in the elevated plus-

maze between Chapters 3 and 4 and Brioni's studies could be due to different brain structures being activated. The low baseline scores in the Brioni studies could indicate that their rats are more stressed than ours are and so the dorsal hippocampus is activated. Further evidence for this is that in animals that were exhibiting an anxiogenic effect after withdrawal from nicotine an injection of nicotine into the hippocampus reversed this effect (Chapter 3), suggesting that the dorsal hippocampus was involved. It would be interesting to investigate whether the response to nicotine changed in animals that were stressed prior to nicotine treatment. If the above hypothesis is correct it would be expected that in stressed animals an anxiolytic effect of nicotine would be seen after systemic and intra-hippocampal injections of nicotine but no effect would be seen after septal administration.

An anxiolytic effect was observed in the elevated plus-maze test in animals that were tested 5 min after the last of 7 days of nicotine treatment. This is in concordance with Pandey et al. (2001) and Ericson et al. (2000) who saw an anxiolytic effect in the plus-maze after 10-14 days of treatment with a higher dose of nicotine. After 7 days of nicotine treatment, tolerance is seen to the anxiogenic effect induced by nicotine (0.1 mg/kg) at 30 min. This would suggest that tolerance has to be seen to the anxiogenic effect of nicotine in this test before the anxiolytic effect emerges. The DRN is a site that is known to mediate the anxiolytic effects in the social interaction test and thus it is possible that it takes a short treatment of nicotine for this brain structure to become activated in this test. This could be investigated by treating the animals for 6 days with systemic nicotine and then on the seventh day, giving an intra-DRN injection of nicotine.

A further difference in these two tests that stands out from the results in Table 10.1 is the time-point at which an anxiogenic withdrawal response is observed after termination of nicotine treatment. In the social interaction test an anxiogenic response is observed at 72 h after nicotine withdrawal whereas in the elevated plus-maze it is seen at 24h. A number of other studies have shown that termination of nicotine causes an anxiogenic withdrawal response (Pandey et al., 2001; Rasmussen et al., 1997; Costall et al., 1989b). In concordance with the result seen in Chapter 3, Pandey et al. (2001) observed an anxiogenic withdrawal response 18h after termination of nicotine treatment. Costall et al. (1989b) observed an anxiogenic response 8-96h after withdrawal from chronic nicotine (0.1 mg/kg/day) in the light-dark exploration test whilst the acoustic startle response was shown to be enhanced on day 1 of withdrawal, peaking on day 4 and back to baseline by day 7 (Rasmussen et al., 1997). Thus, showing that the withdrawal response has a different time course depending on the test of anxiety that is used and therefore suggesting that different types of anxiety are involved at different times after withdrawal from nicotine.

10.2 Time Course of Nicotine's Effects on Anxiety

There is considerable evidence that nicotine can modulate anxiety, but unlike benzodiazepines it does not consistently reduce anxiety. Several factors are crucial in determining the direction of nicotine's effect on anxiety. File et al. (1998) have shown that in the social interaction test the effects of an acute nicotine injection are dose-

dependent, with low doses of nicotine having anxiolytic effects and high doses anxiogenic effects. In the elevated plus-maze, Ouagazzal et al (1999a) have found no effect of nicotine at low doses (0.001 - 0.1 mg/kg) of nicotine, but anxiogenic effects at high doses (0.1 and 0.5 mg/kg). However, other studies using similar doses have shown no effect (0.4 mg/kg) and an anxiolytic effect (0.3 mg/kg) in this test. The results in this thesis have shown that a low dose of nicotine (0.1 mg/kg) has differing effects on anxiety at different times after an acute injection in both the social interaction (Chapter 2) and elevated plus-maze (Chapters 3 and 4; for summary, see Table 10.1) tests. Thus, the effect on an acute dose of nicotine is both dose- and time-dependent.

10.2.1 Effects on anxiety seen 5 minutes after acute nicotine (0.1 mg/kg) injection

Five min after an acute injection of nicotine an anxiogenic effect is seen in the social interaction test (Chapter 2), whereas no effect is seen in the elevated plus-maze at this time point (Chapters 3 and 4). It is known that both the lateral septum and dorsal hippocampus mediate the anxiogenic effects of nicotine in the social interaction test (File et al., 1998; Ouagazzal et al., 1999b). In both cases this anxiogenic effect is reversed by co-administration of a behaviourally inactive dose of the 5-HT_{1A} receptor antagonist WAY 100,635, suggesting involvement of post-synaptic 5-HT_{1A} receptors in both these structures (Cheeta et al., 2000b; Kenny et al., 2000b). The dorsal hippocampus does not seem to play an important role in controlling behaviour on Trial 1 of the elevated plus-maze and administration of nicotine into this area is without effect on Trial 1 of the plus-maze (Ouagazzal et al., 1999a). However, in the elevated plus-maze nicotine is without

effect when administered into the dorsal hippocampus but a significant anxiogenic effect is seen after lateral septal administration (Cheeta et al., 2000b; Ouagazzal et al., 1999b). If the lateral septum were the region activated 5 min after nicotine injection one would expect that the anxiogenic effects would be detected in both the social interaction and elevated plus-maze tests. Thus, it is more likely that the dorsal hippocampus, or some other area not so far explored, such as the basolateral amygdala is the crucial structure involved in mediating this anxiogenic effect in the social interaction test.

The brain regions crucially involved in mediating this short-term effect of nicotine could be investigated by administering mecamylamine, a non-competitive nicotinic antagonist, directly into particular brain regions after systemic nicotine. Once the brain site or sites mediating this anxiogenic effect has been elucidated it would be interesting to attempt to block the anxiogenic effect with nAChR antagonists that are selective for specific nAChR subtypes. The dorsal hippocampus is known to contain a number of nAChR subtypes, predominantly $\alpha4$, $\alpha7$, and $\beta2$ (Léna et al., 1999; Zarei et al., 1999), and therefore the non-competitive antagonists MLA and DHβE, that are selective for $\alpha7$ and $\alpha4\beta2$ respectively, could be used to attempt to block the effect.

The anxiogenic effect in the dorsal hippocampus is due to nicotine causing an increase in 5-HT which then acts on postsynaptic 5-HT_{1A} receptors. Thus, future experiments could investigatė the ability of the 5-HT_{1A} receptor antagonist, WAY 100,635 when it is administered directly into the dorsal hippocampus, to reverse this anxiogenic effect caused by systemic nicotine. It is known that nicotine can increase other

neurotransmitters in the dorsal hippocampus, such as dopamine and noradrenaline (Shim et al., 2001; Kiss et al., 1997; Sershen et al., 1997; Clarke and Reuben, 1996; Sacaan et al., 1996; Toth et al., 1992). Thus, if the effects were not mediated through the 5-HT system it would be interesting to investigate the role of these transmitters by administering antagonists for specific dopamine and noradrenergic receptors into specific brain areas.

Due to the rapidity of the anxiogenic effect a possible mediator of the anxiogenic effect seen in the social interaction test could be corticotrophin-releasing factor (CRF). Central administration of CRF has been shown to have anxiogenic effects in many tests of anxiety, including the social interaction and elevated plus-maze tests (Britton et al., 2000; Spina et al., 2000; Baldwin et al., 1991; Dunn and Berridge, 1990; Dunn and File, 1987). Studies have shown that the effects of CRF are mediated via its binding to two types of receptors, CRF1 receptors (CRF1R) and CRF2 receptors (CRF2R; Chang et al., 1993; Chen et al., 1993). CRF1R knockout mice have shown reduced anxiogenic-like behaviour in animal tests of anxiety compared to wild-type mice (Contarino et al., 1999; Timpl et al., 1998), whereas the knockdown of CRF2 receptors did not result in alterations of anxiety-like behaviours (Heinrichs et al., 1997). Limbic regions, such as the dorsal hippocampus, lateral septum and basolateral amygdala receive a large input of CRF-releasing afferents from the paraventricular nucleus (PVN) of the hypothalamus (Sakanaka et al., 1988a, b; Herman et al., 1996; Kiss et al., 1993). The dorsal hippocampus and basolateral amygdala both have high expression of CRF1Rs and CRF2R, where as the lateral septum has a high expression of CR21Rs (Chalmers et al.,

1995). The effect is most likely through increases of CRF in the dorsal hippocampus as no effect is seen in the elevated plus maze at this time-point. Evidence to support this is that CRF administration into the dorsal hippocampus has shown to have no effect in the elevated plus-maze (Radulovic et al., 1999), whereas an anxiogenic effect is seen after amygdaloid administration (Shepard et al., 2000). The anxiogenic effect is unlikely to be due to an increase in CRF in the lateral septum as no effect is seen in the plus-maze and the receptors in this region are CRF2Rs. Radulovic et al (1999) have demonstrated that stimulation of lateral septal CRF receptors increases anxiety in plus-maze naive animals. However, the dose used in this study was very high and so may have activated the small amount of CRF1Rs that are in this area.

Further experiments could investigate the ability of selective CRF antagonists, such as α-helical CRF_{9-41}, to reverse this anxiogenic effect caused by systemic nicotine. Furthermore, stimulation of 5-HT_{1A} receptors has been shown to increase the release of CRF (Pan and Gilbert, 1992; Kageyama et al., 1998). Therefore, it is possible that the anxiogenic effect of nicotine in the dorsal hippocampus arises because of an increase in the release of 5-HT, which then evokes CRF release by an action at 5-HT_{1A} receptors. If this were the case it would be expected that CRF receptor antagonists would also be capable of reversing the anxiogenic effect.

It is known that in humans acute systemic nicotine cause aversive effects such as gastrointestinal distress, dry mouth, nausea and vomiting (Royal College of Physicians, 2000). Thus, a further possibility for this anxiogenic effect is that the nicotine is acting

peripherally to cause aversive effects that may induce anxiety. In order to determine if this anxiogenic effect is mediated through the CNS, animals could be administered N-methylnicotines that have been shown to poorly penetrate the blood brain barrier (Oldendorf et al., 1993; Aceto et al., 1983). Therefore, if the anxiogenic effect is mediated through the CNS the effect should not be present. Another alternative would be to administer the animals' hexamethonium, a nAChR antagonist that does not cross the blood brain barrier, or mecamylamine prior to nicotine administration. If hexamethonium does not block the anxiogenic effect and mecamylamine does it suggests that the effect is centrally mediated.

10.2.2 Effects on anxiety seen 30 minutes after acute nicotine (0.1 mg/kg) injection

Thirty min after an acute injection of nicotine the results are striking, an anxiolytic effect is seen in the social interaction test but an anxiogenic effect is seen in the elevated plus-maze. This suggests that at this time-point different brain structures are activated in the two tests.

The DRN has been shown to be a neuroanatomical site mediating the anxiolytic effect of nicotine in the social interaction test (Chapter 7). Nicotine stimulates the release of 5-HT in the DRN (Mihailescu et al, 1998), and the reversal of its anxiolytic effect by WAY 100,635 suggest that this action is mediated by indirectly stimulating the somatodendritic 5-HT_{1A} receptors (Chapter 7). Therefore, future experiments could investigate the ability of the 5-HT_{1A} receptor antagonist, WAY 100,635, to reverse this anxiolytic effect when it is administered directly into the DRN after systemic nicotine. The DRN is known to contain nAChRs and therefore it would be interesting to attempt to block the anxiolytic effect with nAChR antagonists that are selective for specific nAChR subtypes, such as

DHβE. The α4β2 sub-type is the most likely candidate as double immunohistochemical labelling has shown the near complete overlap of expression of the serotonin marker tryptophan hydroxylase and the α4 nAChR subunit in the DRN (Bitner et al., 2000). Also, nicotine elicited currents could not be evoked from 5-HT neurones in the DRN in mice lacking both or either of the α4 and β2 sub-units (Cordero-Erausquin et al., 2000).

In contrast to the anxiolytic effect seen in the social interaction test 30min after nicotine injection there is an anxiogenic effect seen in the elevated plus-maze at this time-point. The lateral septum is a site known to mediate the anxiogenic effect of nicotine in this test and therefore the anxiogenic effect seen at this time-point could be due to activation of nAChRs in this area. Therefore, future experiments could again investigate the ability of the 5-HT_{1A} receptor antagonist, WAY 100,635, and nAChR antagonists that are selective for specific nAChR subtypes to reverse this anxiogenic effect. There is little in the literature on the nAChR subtypes that are in the lateral septum but as this site receives a huge input from the dorsal hippocampus it is likely that similar nAChR subtypes are present. Thus, administration of MLA and DHβE may be possible antagonists for blocking this effect. One study did find that there was a high amount of [^{3}H]-cytisine binding in this area (Davila-Garcia et al., 1999). As cytisine has high affinity for the β4 subtype it would suggest that these are the present. At this point in time there is no receptor antagonist that is highly specific for this sub-unit.

10.2.3 Effects on anxiety seen 60 minutes after acute nicotine (0.1 mg/kg) injection

Sixty min after an acute injection of nicotine an anxiogenic effect is observed in the social interaction test but no effect is seen in the elevated plus-maze. After systemic administration of nicotine maximal amounts of nicotine are seen in the brain 30-60 min later (Crooks and Dwoskin, 1997). Therefore, it may take until 60 min after nicotine administration when the nicotine concentration in the brain is at its highest for activation of a particular nAChR to occur. Thus, the anxiogenic effect could again be due to activation of nAChRs in the dorsal hippocampus or lateral septum increasing 5-HT release. If the dorsal hippocampus were mediating the initial anxiogenic effect seen at 5 min it would be unlikely that it is also mediating the anxiogenic effect seen at 60 min. Thus, the lateral septum would appear to be the likely candidate. However, again at this time-point there is no effect seen in the plus-maze. In contrast, to the effects seen in the plus-maze 5 min after injection, higher doses (0.25-0.45 mg/kg) do induce an anxiogenic effect 60 min after injection. The anxiogenic effect seen after later septal administration appears to be greater in the social interaction test (81% after 4μg) than in the plus-maze (30% after 4μg) and thus, 0.1 mg/kg nicotine may be high enough to cause an anxiogenic effect in the social interaction test but not in the elevated plus-maze at this time-point.

Therefore, the brain regions involved in mediating this late effect of nicotine could be investigated by administering mecamylamine directly into particular brain regions after systemic nicotine. Again, after finding the brain site mediating this effect attempts could

be made to block the anxiogenic effect with nAChR antagonists that are selective for specific nAChR subtypes. As the anxiogenic effect in the lateral septum is due to nicotine causing an increase in 5-HT, future experiments could investigate the ability of the 5-HT_{1A} receptor antagonist, WAY 100,635, to reverse this anxiogenic effect caused by systemic nicotine.

Another possibility is that the anxiogenic effect could be mediated by another neurotransmitter. A stimulant effect on locomotor activity is observed 60 min after an acute injection of 0.1 mg/kg nicotine (Clarke and Kumar, 1983a, b) and this is thought to be due to dopamine. Furthermore, dopamine release has been shown to be increased in certain brain areas 60 min after injection (Shim et al., 2001). Therefore, further experiments could investigate whether administering antagonists for specific dopamine receptors into specific brain areas could reverse the anxiogenic effect seen 60 min after nicotine.

For more details of the mechanisms that mediate the complex time course in social interaction see Appendix 1.

10.3 Tolerance to Nicotine's Effects on Anxiety

One of the main themes of this thesis has been investigating the development of tolerance to nicotine's effects on anxiety. Tolerance is a reduced sensitivity to the behavioural effects of a drug following repeated administration and is thought to involve multiple adaptive processes (Young and Goudie, 1995). There are thought to be two different types of tolerance, dispositional (pharmacokinetic) and functional (pharmacodynamic) tolerance. Dispositional tolerance results from changes in absorption, distribution or

metabolism of a drug, whereas functional tolerance is usually taken to be tolerance that is mediated by changes in the sensitivity of the neuronal, receptor or neurochemical system which may limit a drug's actions (Goudie, 1989). It has been suggested that functional tolerance may be made of two distinct processes, decremental and oppositional (Littleton and Little, 1989). A decremental adaptation is described as a response in a system resulting in a decreased effect on the drug system but as this adaptation would only be apparent in the presence of the drug, there would not necessarily be any functional indication in the absence of the drug. Oppositional refers to an activation of an oppositional mechanism within a system to counteract the drug effect and in this case, withdrawal responses would be a manifestation of the functional disturbance created by an oppositional adaptation that is not counteracted by the presence of the drug. When considering the development of tolerance, it is important to remember that it is possible for multiple processes to be involved (Young and Goudie, 1995).

10.3.1 Tolerance to the low dose of nicotine

In the social interaction test, tolerance was found to occur to both the anxiogenic and anxiolytic effects that were observed 5 and 30 min after acute nicotine administration, respectively, after 7 days of nicotine treatment (Chapter 2). This is consistent with the time course of tolerance to other behavioural effects of nicotine, such as anti-nociception and milk intake (McCallum et al., 1999; Wewers et al., 1999). The DRN and dorsal hippocampus were found to be brain structures involved in mediating tolerance to these anxiolytic and anxiogenic effects, respectively (Chapters 7 and 8). Tolerance to the

anxiolytic effect is thought to be due to an oppositional mechanism as an anxiogenic withdrawal response is seen 72h after termination of nicotine treatment. The mechanism that mediates this tolerance is thought to be due to a compensatory change such as increased firing of the raphé neurones, which would lead to increased 5-HT release in limbic areas when the nicotine is withdrawn. A similar mechanism in the median raphé nucleus has been shown to mediate the anxiogenic response that occurs on withdrawal from chronic benzodiazepine treatment (Andrews et al, 1997). However, tolerance to the anxiogenic effect is thought to be due to a decremental mechanism as a significant attenuation of the ability of nicotine to stimulate 5-HT release in the dorsal hippocampus after chronic treatment is observed, which could have arisen as a result of receptor desensitisation (Radcliffe et al., 1999; Alkondon et al., 1997). Administration of nicotine into the lateral septum had an anxiogenic effect in the social interaction and elevated plus-maze tests. Thus, it would be interesting to investigate if tolerance is seen to the anxiogenic effect in these tests after 7 days of nicotine treatment. The anxiogenic effect seen in both the tests after lateral septal administration is blocked by the 5-HT_{1A} receptor antagonist, WAY 100,635, suggesting that nicotine increases 5-HT release which then acts on postsynaptic 5-HT_{1A} receptors. Thus, future experiments could investigate the effect of nicotine on 5-HT release from this brain site in animals that were drug naive and had been chronically treated with nicotine. This could either be done using in vitro slice release or a better technique would be to use in vivo microdialysis.

Interestingly, after 6 days of nicotine treatment tolerance is observed to the anxiolytic effects of midazolam in both the dorsal hippocampus and DRN (Chapter 9). This result

is very striking as the development of tolerance is much more rapid than the tolerance that develops after chronic benzodiazepine treatment, which usually takes approximately 3 weeks to develop, thus showing that nicotine treatment can modify it's own behavioural effects and those of the benzodiazepines. It would be interesting to do the reverse of this study and investigate if chronic benzodiazepine treatment altered nicotine's effects on anxiety. Chronic nicotine also decreased [^{3}H]-flunitrazepam binding in the dorsal hippocampus and therefore, a further study would be to investigate if there was a change in nicotinic receptors after chronic benzodiazepine treatment.

10.3.2 Why is tolerance so slow to the high dose of nicotine?

After 4 weeks of treatment with a high dose of nicotine (0.45 mg/kg/day) complete tolerance does not develop to the anxiogenic effects and this is consistent between the different routes of administration that were used (Chapters 5 and 6). This is in contrast to the rapid rate of development of tolerance to the low dose effects of nicotine on anxiety and the tolerance to nicotine's effects on anti-nociception (0.35 mg/kg) and milk intake (0.66 mg/kg) after treatment with a high dose for 7 days (McCallum et al., 1999). It is also surprising that no tolerance was seen to the anxiogenic effects of this high dose of nicotine as tolerance normally develops faster when a higher dose is administered. Indeed, tolerance to the acute stimulatory effects of caffeine were seen faster with a higher dose than with a lower dose (Gasior et al., 2000). File et al. (1998) showed that there was no difference in the anxiogenic response after 0.5 and 1.0 mg/kg nicotine suggesting at a certain anxiogenic dose there is a floor effect of nicotine which cannot be

augmented by increasing the nicotine dose and thus nicotine may only be able to modify anxiety within certain limits, although pharmacologically it may be having a much greater effect. Therefore, the high dose of nicotine used in Chapters 5 and 6 may cause a paharmacological change, such as alteration in the homeostatic balance of the serotonergic and cholinergic systems, and tolerance has to be seen to this alteration before a change is observed in the behaviour. Therefore, it may be, and is likely, that longer treatment with this dose would have resulted in complete tolerance. This could be investigated further by treating the rats for a longer period of time and testing them for tolerance.

It is also possible that different mechanisms of tolerance have to be recruited to see tolerance to the anxiogenic effects of this high dose. There is now considerable evidence for the role of learning as an adaptive process influencing the development of tolerance arising from an individual's experiences in the drugged state (Goudie and Griffiths, 1996; Young and Goudie, 1995). A process thought to be important in the mediation of drug tolerance is one in which an individual learns to deal with the effect of the drug, resulting in acquired behavioural skills for coping with the drug-induced effect. Although tolerance resulting from a learned coping ability could involve a non-specific strategy and be situation independent (Demellweek and Goudie, 1983), it could theoretically be augmented by "practising" in the presence of the drug stimulus and possibly by association with the environment in which the individual practices the coping strategy (King et al., 1987). However, it is unlikely that learned tolerance plays a role in tolerance to the anxiogenic effect of this high dose of nicotine as the same effects were

seen in the animals that received nicotine via minipumps where the drug is not paired to anything and has no clear onset of action. It is unlikely that metabolic tolerance is playing a part as there is no difference in plasma nicotine levels between the animals that were treated for 4 days or 4 weeks with nicotine by subcutaneous injections or continuous infusion by osmotic minipump.

10.3.3 Why is there no withdrawal from the high dose of nicotine?

Termination of nicotine treatment results in an anxiogenic withdrawal that has been observed in a number of animal tests of anxiety (Pandey et al., 2000; Rasmussen et al., 1997; Costall et al., 1989b). Termination from one week of treatment with a low dose of nicotine results in an anxiogenic withdrawal response that is seen at 24h in the elevated plus-maze (Chapter 3) and 72h in the social interaction (Chapters 2 and 8) tests. However, in animals receiving a higher dose of nicotine (0.45 mg/kg/day) for 4 weeks no anxiogenic withdrawal response was observed at either the 24 or 72h time-point (Chapter 5 and 6). This was again irrespective of treatment regimen. This lack of withdrawal response at these two time-points is very surprising but there are possible reasons to explain it. The simplest reason is that the wrong time-points after termination of nicotine treatment were used to test the rats following this particular dose-regime. Results from other studies have shown that an anxiogenic withdrawal response can be seen at time-points ranging from 18h to approximately 4-5 days (Chapters 2, 3 and 8; Pandey et al., 2000; Rasmussen et al., 1997; Costall et al., 1989b). In order, to investigate this possibility a more detailed study needs to be conducted in which an

increased number of time-points after termination of nicotine treatment are used. Another possible explanation is that because the animals had not developed complete tolerance to the anxiogenic effect of nicotine no significant withdrawal changes had yet developed. The withdrawal response observed in the social interaction test after a low dose of nicotine is thought to be due to an oppositional mechanism of tolerance to the anxiolytic effect. It was suggested that the delayed anxiolytic effect observed in the elevated plus-maze after 7 days of treatment was due to the need for tolerance to the anxiogenic effect to be seen before an anxiolytic effect emerged. Therefore, in the case of the higher dose of nicotine, tolerance may have to be seen to the anxiogenic effect of nicotine before the anxiolytic effect emerges which could then cause an oppositional mechanism that would result in a withdrawal response.

10.4 Implications for Smoking

Many smokers report cigarettes have an anxiety reducing influence and that mood control is a core reason for maintaining their smoking habit (Parrot, 1994). Most of the theories concerning the role of anxiety states in smoking behaviour have focussed on the putative anxiolytic effects. Indeed, smokers frequently report calming and anxiety reducing effects (Ikard et al., 1969). There is evidence that during episodes of stress and depression smoking increases (Breslau et al., 1991). Furthermore, a recent report demonstrated that high yield nicotine, but not low yield nicotine, cigarettes reduce subjective anxiety in smokers during stressful situations suggesting that nicotine has dose-dependent effects on humans (Kassel and Unrod, 2000). However, there is

evidence that not all smokers experience anxiolytic effects from smoking (Parrott and Garnham, 1998) and in general it seems that smoking has anxiolytic effects when smokers are exposed to moderate levels of stress/anxiety, but is ineffective when there is no stress (Gilbert and Wessler, 1989). Furthermore, it has been reported by Newhouse et al. (1990) that smokers with high neuroticism scores become more anxious and tense after smoking a cigarette. The results from this thesis showing that nicotine can have both acute anxiolytic and anxiogenic effects would lend further weight to a likely bi-directional effect of smoking on anxiety. It is unclear what the role of these acute effects of nicotine would have in the onset of smoking, as there are a number of different factors that could influence this, such as stress and peer pressure. However, it is likely that anxiety has an important role in the maintenance of smoking. Chapter 5 showed that an increase in anxiety was observed in rats chronically self-administering nicotine, compared with rats chronically self-administering saline. This suggests that a continuing anxiolytic effect is not necessary for the maintenance of nicotine self-administration and that the rats may actually self-administer nicotine precisely for its ability to produce an increase in anxiety, and a very similar argument has been proposed for the rewarding effects of cocaine (Goeders, 2001). This persisting anxiogenic effect was also observed in animals that received the same dose of nicotine by intravenous and subcutaneous injections or subcutaneous infusion (Chapter 6). This suggests that the route of administration of nicotine may be of less importance for the role that anxiety plays in maintaining smoking behaviour.

It is the general consensus that an increase in anxiety is seen in nicotine withdrawal in smokers (Shiffman and Jarvik, 1976; West and Russell, 1985) and those using nicotine gum (Hughes et al., 1990; Keenan et al., 1989; Wesnes and Warburton, 1983). Nicotine withdrawal symptoms peak within the first week of abstinence and can last up to 4 weeks (Jorenby et al., 1996; Hughes et al., 1990). Nicotine patches and tablets have been shown to reduce anxiety in smokers and this has been suggested to be due to a reversal of withdrawal anxiety (Netter et al., 1998; Warburton and Mancuso, 1998; Wesnes and Warburton, 1983). Indeed, it has been proposed that the anxiolytic effects of nicotine observed in smokers arises only because nicotine reverses the withdrawal-reduced anxiety (Parrott, 1999). The studies reported in this thesis also show an anxiogenic response accompanies nicotine withdrawal in rats and can be reversed by nicotine. This further suggests a role for anxiety in the maintenance of smoking and provides an animal model for investigating the neurobiological mechanism involved in smoking behaviour and relapse.

Both in the human and animal literature there has been a lack of attention paid to the sex differences seen in nicotine's response to nicotine, but a recent review (Perkins et al., 1999) highlights the potential importance of this. There is accumulating evidence to suggest that males and females may differ in factors that maintain tobacco smoking and nicotine self-administration. The effects of nicotine in non-smokers are particularly relevant to the issue of smoking initiation. In a recent study conducted on non-smoking 20 year-olds the effects of sex differences in response to nicotine were examined (File et al., 2000a). After exposure to a moderately stressful situation, nicotine blocked the

increase in anxiety and discontent and reduced aggression in females, but not males. This provides important evidence that there are sex differences in the anxiolytic effects of nicotine. All the experiment conducted in this thesis have been conducted in adult male rats and therefore it would be interesting to investigate the effects of nicotine treatment in female rats in both the social interaction and elevated plus-maze tests of anxiety. Perhaps though a more appealing study would be to investigate the effects of nicotine in both adolescent male and female rats. To date, there is no existing data on the response to nicotine in behavioural tests using adolescent rats. This would be particularly pertinent as adolescent smokers report significantly higher levels of nervousness, stress and anxiety than do age-matched non-smokers (Lloyd and Lucas, 1997; Wills, 1986; Mitic et al., 1985; Hirschman et al., 1984) and 64% of adolescent female daily smokers report that they feel calmer after smoking (McNeill et al., 1997).

10.5 Future Research

Chapter 9 showed that after just 6 days of systemic nicotine treatment tolerance was seen to the anxiolytic effects of midazolam when it was injected directly into the dorsal hippocampus and the DRN. It would be interesting to investigate if cross-tolerance was seen between nicotine and other psychostimulants, such as cocaine and caffeine. Horger et al. (1992) showed that the acquisition of low-dose intravenous cocaine self-administration was more rapid in animals that had been prexposed to nicotine compared to controls, thus suggesting that cocaine may show cross-tolerance with nicotine. In contrast, a recent study showed that there was no cross-tolerance to caffeine in animals that were tolerant to the stimulant effects of nicotine (Domino et al., 2001).

10.6 Conclusion

In conclusion, the studies in this thesis have high-lighted that the effects of nicotine on anxiety are dependent on a number of factors including the dose of nicotine, the chronicity of drug treatment and the animal test of anxiety used. Furthermore, different neuroanatomical substrates have been shown to play distinct roles. Taken together these studies highlight the very complex role that anxiety plays in smoking and one that may have been overlooked in the past.

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APPENDIX 1

The time course of nicotine's effects on anxiety in the social interaction test

The results of the experiments in Chapter 2 show that there is a complex pattern of change in social interaction induced by nicotine. Anxiogenic effects are seen 5 and 60 min after injection and an anxiolytic effect 30 min after injection. It is thought that these different effects are mediated by nicotine's action in different neuroanatomical sites. The initial 5 min effect may be due to nicotine acting at α7-type nAChRs in the dorsal hippocampus, a known anxiogenic site for nicotine (File et al., 1998). It is known that this receptor sub-type predominates in this area (Zarei et al., 1997). Activation of this nAChR is then thought to cause an increase in 5-HT release that acts on post-synaptic 5-HT_{1A} receptors and elicits an anxiogenic effect in the social interaction test (File et al., 2000b; see Figure A1.1). However, this receptor sub-type is known to undergo rapid desensitisation whereas the α4β2 is known to desensitise at a slower rate (Gerzanich et al., 1994). It is possible that the anxiolytic effect seen 30 min after injection is due to nicotine's action at α4β2-type nAChRs in the DRN, a known anxiolytic site for nicotine (Chapter 7). There is much evidence to suggest the DRN contains this receptor subtype in high abundance (Bitner et al., 2000; Cordero-Erausquin et al., 2000). This anxiolytic effect is thought to be due to nicotine causing an increase in 5-HT release in the DRN that acts on somatodendritic 5-HT_{1A} receptors leading to a reduction in 5-HT neuronal firing, and a subsequent decrease in 5-HT release in terminal regions of the limbic system (Chapter 7; see Figure A1.1). The anxiogenic effect seen 60 min after injection may be mediated by the action of nicotine in the lateral septum. Like the dorsal hippocampus the lateral septum is known to mediate the anxiogenic effects of nicotine in the social

interaction test by activating a presynaptic nAChR on a 5-HT neurone causing an increase in 5-HT which then activates post-synaptic 5-HT_{1A} receptors (Ouagazzal et al., 1999b). However, it is unknown which nAChR sub-types that are in the lateral septum. If it is this structure that mediates the anxiogenic effect seen 1h after nicotine injection then these pre-synaptic nAChRs must either have a low affinity for nicotine and only become activated when maximal amounts of nicotine are seen in the brain, or they must desensitise much slower than the two aforementioned nAChR sub-types.

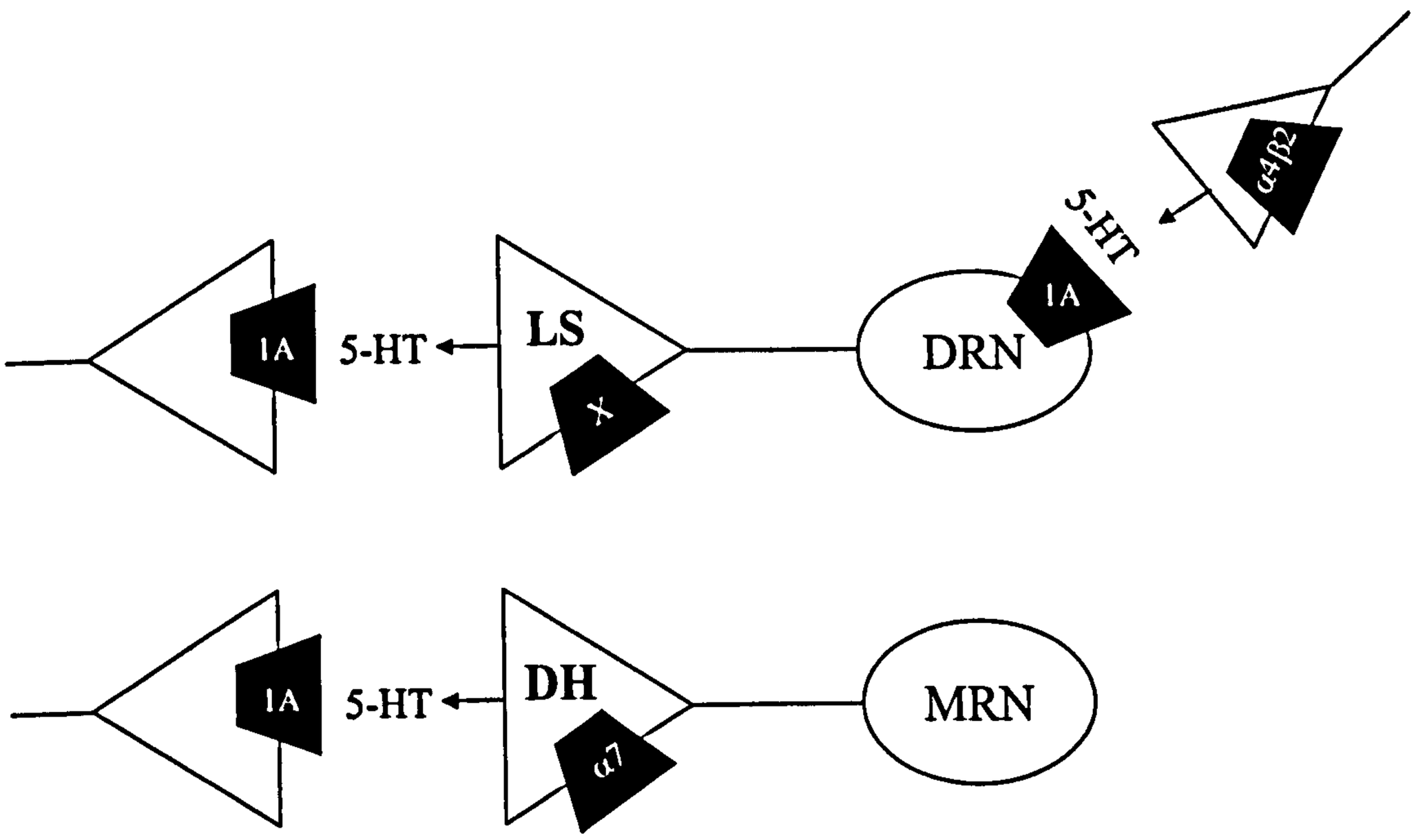

Figure A1.1 Proposed mechanisms for nicotine's effects on anxiety in the social interaction test. DRN, Dorsal Raphé Nucleus; MRN, Median Raphé Nucleus; LS, Lateral Septum; DH, Dorsal Hippocampus; α7, α4β2-type nAChR; α4β2, α4β2-type nAChR; X, nAChR sub-type unknown; 5-HT, Serotonin; 1A, 5-HT_{1A} receptors.

APPENDIX 2

Effects of chronic diazepam treatment on [^{3}H]nicotine binding

A2.1 Introduction

Benzodiazepines and nicotine are drugs that are abused by many individuals and it is thought that there may be a link between the mechanisms involved in their dependence. There is evidence to suggest that people dependent on benzodiazepines are significantly more likely to smoke than people who are not (Lekka et al., 1997). Further evidence for cross tolerance between these two drugs is seen in Chapter 9 where after only 6 days of treatment with a low dose of nicotine tolerance is seen to the anxiolytic action or midazolam. The main action of the benzodiazepines appear mainly to involve the GABAergic system in the brain as they bind to a site on the $GABA_A$ receptor allosterically modulating it. However, there have been some indications in the literature that the cholinergic system may also be involved in the action of benzodiazepines (Skolnick and Paul, 1981). Benzodiazepines have been shown to increase the content of ACh in various brain areas of animals (Consolo et al., 1974; Bianchi et al., 1975) and to decrease the turnover of ACh (Zsilla et al., 1976). It has been shown that in rats treated with diazepam for 3 weeks there are changes in both the number and affinity of the muscarinic receptors in the brain (Popova et al., 1988). There appears at present to be no literature examining whether chronic treatment with benzodiazepines has alters these changes in nicotinic receptors in the brain.

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To study whether there is a possible link between benzodiazepines and nicotinic receptors during benzodiazepine dependence, the aim of this work was to investigate the effect of chronic diazepam (15 mg/kg/day, s.c.) exposure on the number of nicotinic receptors in the frontal cortex and cerebellum of rat. The first part of the study involved conducting a number of small experiments to optimise the conditions for the nicotinic acetylcholine binding site assay. Unfortunately, for the main part of the study only the cerebellar tissue was investigated due to the frontal cortex homogenates becoming contaminated with bacteria.

A2.2 Materials and Methods

Animals

Brain tissue was obtained from the animals used in the previous chapter that were treated with daily sub-cutaneous injections of vehicle, acute diazepam or 7, 14 or 28 days of diazepam (15 mg/kg/day). Animals were sacrificed immediately after testing by decapitation, and the brains immediately removed, dissected, frozen in an ethanol dry ice bath and stored at -20°C until required. Brain tissue for optimising the assay conditions came from untreated animals that were killed the same way.

Membrane Preparation

Frozen brain tissue was thawed at room temperature and homogenised in ice-cold 0.32M sucrose using an Ultra-Turex homogeniser. The homogenate was centrifuged at 1000g (10 min, 4°C) to remove myelin and cell debris. The pellet was discarded and the supernatant was then centrifuged at 15000g (20 min, 4°C). The pellet was resuspended in 10ml of 50mM Tris-HCl buffer containing 120mM NaCl and 5mM KCl, pH 7.4 (referred to as Tris-HCl plus

salts) and centrifuged at 15000g (20 min, 4°C). The latter process was repeated a further two times increasing the volume of Tris-HCl plus salts to 20 and 30mls, respectively. The final pellet was resuspended in a volume of Tris-HCl plus salts to give a concentration of 60 mg wet weight tissue/ml. Samples of each membrane preparation were stored at -20°C for determination of protein content.

Receptor Binding Assay

Drugs and radioligand were prepared in 50mM Tris-HCl plus salts, pH 7.4. Assays were performed in Macrowell 1.4ml tubes (Helios Biotech). Aliquots of membrane preparation (475µl; approximately 75µg protein), 5µl [^{3}H]nicotine (5nM) and 20µl of either Tris-HCl plus salts to define total binding or 100µM (final) nicotine to define non-specific binding (final volume 500µl) were incubated at room temperature for 30 min and then at 4°C for 1h. Total and non-specific binding were both performed in triplicate.

Termination of Incubation

Bound and free ligand were separated by vacuum filtration with a cell harvester (Skatron AS) through Whatman GF/C glass fibre filters, previously treated with 0.01% polyethylenimine to inhibit non-specific binding. Filters were washed for 15 s with ice-cold 50mM Tris-HCl plus salts buffer, pH 7.4.

Scintillation Counting

The filters were placed in Pico Pro scintillation vials with 5mls Packard Emulsifier safe scintillation fluid and thoroughly mixed. The amount of radioactivity per tube was determined

using a Wallac WinSpectral (Model 1414) scintillation counter programmed to count for 5 min per sample at an efficiency of 42-43%. Counts per minute (cpm) were converted to disintegrations per min (dpm) using external standardisation.

Protein Assay

Protein concentrations were assessed in triplicate using Comassie Plus protein assay reagent (Pierce) and measuring absorbance at 595nm. Bovine serum albumin was used as the protein standard.

Chemicals

L-[^{3}H]nicotine (N-methyl-[^{3}H]; 81.5 Ci/mmol) was purchased from DuPont, NEN (Stevenage, UK). (-)-nicotine hydrogen tartrate, sucrose, Tris HCl, Tris Base and KCl were purchased from Sigma (Poole, Dorset, UK). NaCl was purchased from BDH (Poole, Dorset, UK).

Statistics

The % specific binding was analysed by one-way analysis of variance and comparisons between individual groups were then made with the LSD (least significance difference) post-hoc test.

A2.3 Results

Initially, a series of experiments was conducted to determine the optimal conditions for the nicotinic acetylcholine binding site assay. Membrane preparation and binding assays were carried out as described above, unless otherwise stated.

Determination of the Optimal Protein Concentration in the Frontal Cortex

The binding of [^{3}H]nicotine to frontal cortical homogenates was investigated using protein concentrations of 125, 100, 75, 50, 37.5, 25 and 12.5 µg/assay. The results are summarised in Table A2.1.

Table A2.1 The effect of varying tissue concentration on [^{3}H]nicotine binding to membranes prepared from frontal cortical homogenates. Results are expressed in % specific binding ± sem.

Protein Concentration (µg/assay)	% Specific Binding	n
125	58.3 ± 1.2	3
100	36.0	2
75	50.5 ± 3.8	6
50	29.0	2
37.5	28.2 ± 4.3	4
25	20.5	2
12.5	14.8 ± 8.6	4

A tissue concentration of 75µg/assay was chosen for further studies. Although 125 µg/assay showed the greatest percentage of specific binding this was not significantly different from 75 µg/assay and so in order to minimise the amount of tissue used the above concentration was decided on.

Determination of the Optimal Ligand Concentration and Incubation Method in the Frontal Cortex

The binding of [^{3}H]nicotine to frontal cortical homogenates was investigated using ligand concentrations of 5, 7.5 and 10nM using a 2h incubation at 4°C (Method 1) or incubation at room temperature for 30 min and then placed at 4°C for 1h (Method 2). The results are summarised in Table A2.2.

Table A2.2 The effect of varying tissue concentration on [^{3}H]nicotine binding to membranes prepared from frontal cortical homogenates. Results are expressed in % specific binding ± sem.

	% Specific Binding	
Ligand Concentration (nM)	Method 1	Method 2
10	43.5 ± 5.5	36.5 ± 3.5
7.5	21.0 ± 4.0	43.5 ± 1.5
5	25.0 ± 8.0	35.5 ± 9.5

A ligand concentration of 5nM was chosen in order to minimise the amount of ligand used and therefore the cost. There was no difference between the methods of incubation and so

method 2 was chosen as there seemed to be lower non-specific binding with this method than method 1 for each of the ligand concentrations used.

Determination of the Optimal Protein and Ligand Concentration in Cerebellar Tissue

The binding of [^{3}H]nicotine to cerebellar homogenates using protein concentrations of 100, 75, 50, and 25µg/assay using 2 ligand concentrations, 5 and 10nM, were investigated. The results are summarised in Table A2.3.

For the cerebellar homogenates 75µg/assay and 5nM were chosen as the respective protein and ligand concentration in order to minimise the amount of tissue and ligand needed.

Table A2.3 The effect of varying tissue and ligand concentration on [^{3}H]nicotine binding to membranes prepared from cerebellar homogenates. Results are expressed in % specific binding ± sem.

	Ligand Concentration (nM)	
Protein Concentration (µg/assay)	5	10
100	52.0 ± 10.0	46.5 ± 9.5
75	52.5 ± 1.5	37.0 ± 19.0
50	43.5 ± 8.5	46.0 ± 5.0
25	37.0 ± 4.0	48.0 ± 1.0

The Effect of Duration of Diazepam Treatment on [^{3}H]nicotine Binding in Cerebellar Homogenates

There was a significant effect of duration of diazepam treatment on [^{3}H]nicotine binding in cerebellar tissue [$F(4,12)=3.3$, $p<0.05$); Figure A2.1]. Acute administration of diazepam was not significantly different from vehicle controls. Post-hoc tests showed that following 7 days treatment of diazepam there was a significant decrease in the % specific binding compared to the vehicle and acute diazepam groups ($p<0.05$ for both groups). After 14 days of diazepam treatment there was still a significance decrease compared to the vehicle group ($p<0.05$) but there was no longer a difference from the acute group. After 28 days diazepam treatment there was no significant change from any of the groups.

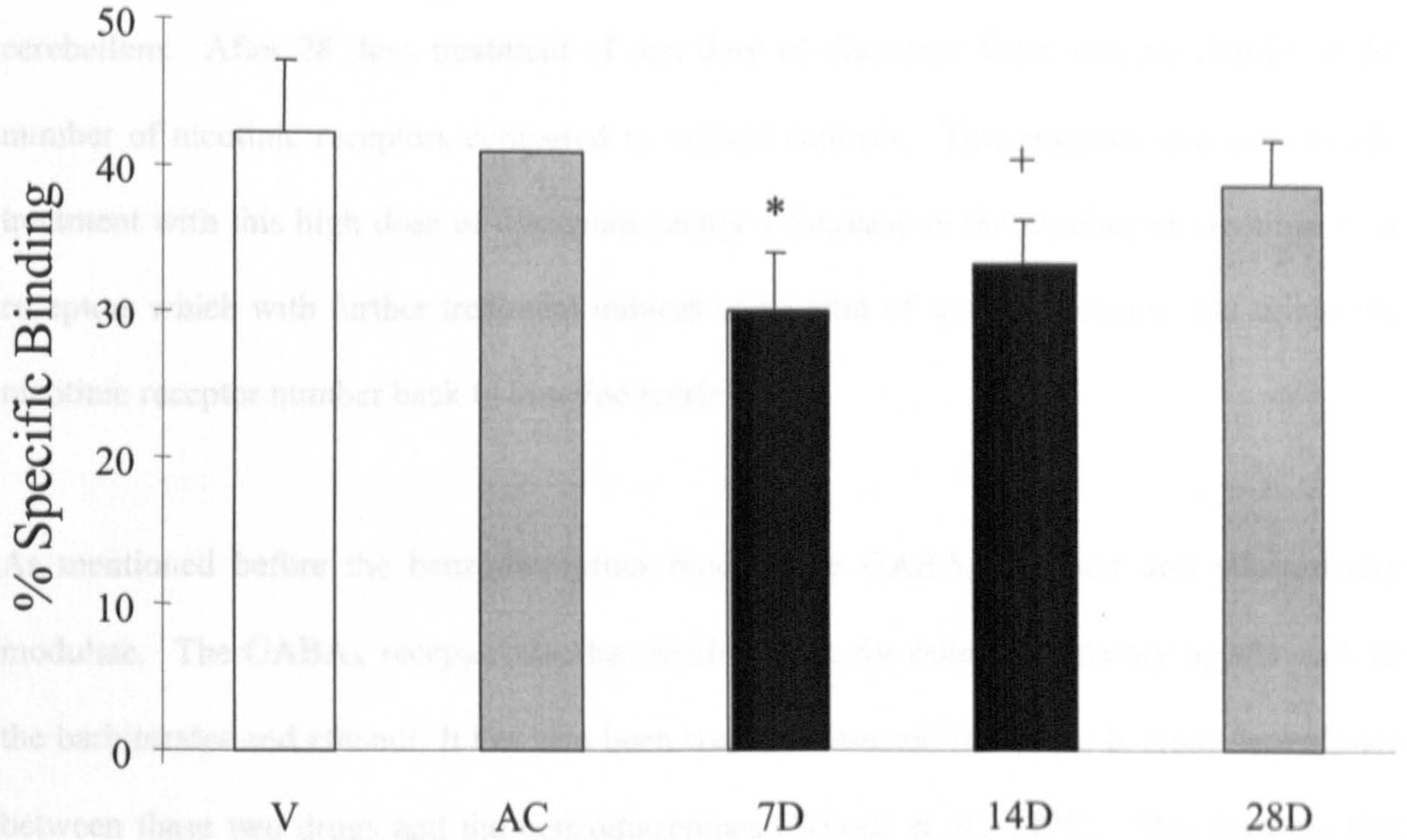

Figure A2.1 Rats were treated with daily sub-cutaneous injections of vehicle (n=5), acute (n=2) diazepam or 7 (n=4), 14 (n=3) or 28 (n=3) days of diazepam (15 mg/kg/day). The nAChR density in cerebellar homogenates was measured by [^{3}H]nicotine binding and results are expressed in % specific binding ± sem. *$p<0.05$ compared with vehicle control and acute group and $^+p<0.05$ compared with vehicle control

A2.4 Discussion

The initial series of experiments showed that to obtain optimal binding a concentration of 75 µg/assay of tissue should be incubated with 5nM [^{3}H]nicotine for 30 min at room temperature and then for a further hour at 4°C. The main part of this study showed there was a significant

decrease in the number of nicotinic receptors following 7 and 14 days treatment with diazepam (15 mg/kg/day; s.c.) measured with [^{3}H]nicotine as the radioligand in the cerebellum. After 28 days treatment of this dose of diazepam there was no change in the number of nicotinic receptors compared to vehicle controls. This suggests that sub-chronic treatment with this high dose of diazepam causes a decrease in the number of nicotinic ACh receptors which with further treatment induces some kind of adaptive process that brings the nicotinic receptor number back to baseline levels.

As mentioned before the benzodiazepines bind to the $GABA_A$ receptor and allosterically modulate. The $GABA_A$ receptor also has binding sites for other modulatory agents such as the barbiturates and ethanol. It has also been well documented that there is cross-dependence between these two drugs and the benzodiazepines (Woods et al., 1990). This suggests that information known about the actions of these two drugs on the cholinergic system might be useful in determining the mechanism of action of the benzodiazepines. As for the benzodiazepines it is known that both the barbiturates and ethanol increase endogenous acetylcholine in various brain areas and increases the number of muscarinic receptors. It is known that the barbiturates bind to both muscarinic and nicotinic receptors but to date this has not been observed for ethanol or benzodiazepines (Nordberg and Wahlström, 1992). Therefore, it is possible that diazepam may bind to a site on the nicotinic ACh receptor that is as yet unknown. It is well reported that chronic treatment with nicotine results in an increase in the number of nicotinic receptors in the brains of both humans (Benwell et al., 1988) and animals (Collins et al., 1994, 1990, 1988; Marks et al., 1985, 1984, 1983). This upregulation is thought to occur due to desensitisation or inactivation of the nicotinic receptors. Chronic treatment with diazepam could increase the release of ACh and/or bind directly to the nicotinic receptors causing an initial decrease in the number of nicotinic receptors. Over time

these two possible effects could begin to cause desensitisation or inactivation of theses receptors causing the numbers to increase. Thus, if the treatment with diazepam was continued for a further few weeks a possible increase in these receptors could be seen.

In conclusion, this study has shown that chronic treatment with diazepam can produce decreases in the number of nicotinic receptors in the cerebellum, which after a longer period of time go through an adaptive process bringing them back to normal levels. Further work is warranted into the search to discover whether there is a link between the benzodiazepines and nicotine in dependence. It would also be interesting to look in areas of the brain that are believed to be involved in anxiety to try and elucidate whether there is a link between the tolerance mechanisms to anxiety for these two drugs.